Johanna Eerola-Rautio

SPECT-AIDED DIAGNOSTICS AND GENETIC RISK FACTORS IN PARKINSON'S DISEASE

Department of Neurology
Helsinki University Central Hospital

Research Program of Molecular Neurology
Biomedicum Helsinki
University of Helsinki

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SUPERVISOR

Docent Pentti J. Tienari

Department of Neurology

Helsinki University Central Hospital

Research Program of Molecular neurology, Biomedicum Helsinki

University of Helsinki

REVIEWERS

Kari Majamaa

Professor of Molecular Medicine

University of Oulu and

Professor of Neurology (on leave)

University of Turku

Docent Mikko Kuoppamäki

Clinical Research and Development, CNS

Orion Pharma

OPPONENT

Professor Vilho Myllylä

Department of Neurology

University of Oulu

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CONTENTS

LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following publications. They are referred to in the text by their roman numerals.

I Eerola J, Tienari PJ, Kaakkola S, Nikkinen P, Launes J. How useful is ^{123}I-β-CIT SPECT in clinical practice? J Neurol Neurosurg Psychiatry 76(9):1211-1216, 2005.

II Eerola J, Launes J, Hellström O, Tienari PJ. *APOE*, *PARKIN* and *COMT* genes and susceptibility to idiopathic Parkinson's disease in Finland. Neurosci Lett 330:296-298, 2002.

III Eerola J, Hernandez D, Launes J, Hellström O, Hague S, Gulick C, Johnson C, Hardy J, Tienari PJ, Singleton AB. Assessment of a DJ-1 (PARK7) polymorphism in Finnish PD. Neurology 61(7):1000-1002, 2003.

IV Luoma PT, Eerola J, Ahola S., Kivisto K, Hellström O, Tienari PJ, Suomalainen A. Mitochondrial DNA polymerase gamma (*POLG1*) variants in idiopathic sporadic Parkinson disease. Neurology 69:1152-1159, 2007. *

V Eerola J#, Luoma P#, Peuralinna T, Scholz S, Paisan-Ruiz C, Suomalainen A, Singleton AB, Tienari PJ. Rare POLG1 polyglutamine tract variants associated with Parkinson's disease. Submitted.

In addition, some unpublished data are presented.

* Article IV also appeared in PhD Petri Luoma's thesis in 2007
\# equal contribution

ABBREVIATIONS

AAO	age-at-onset
AD	autosomal dominant
ALP	autophagy-lysosome pathway
APOE	apolipoprotein E
AR	autosomal recessive
β-CIT	2 β-carbomethoxy-3β-(4-iodophenyl)tropane
bp	base pair
CBD	cortico-basal degeneration
CMA	chaperone-mediated autophagy
COMT	catechol-o-methyltransferase
DA	dopamine
DAT	dopamine transporter
DBS	deep brain stimulation
DIP	drug-induced parkinsonism
DLB	dementia with Lewy bodies
DNA	deoxyribonucleic acid
EOP	early-onset parkinsonism
GWA	genome-wide association
GST	glutathione transferase
H&Y	Hoehn & Yahr scale
IBZM	Iodobenzamide
kb	kilobase
LB	Lewy body
LD	linkage disequilibrium
L-DOPA	levodopa
MAPT	microtubule-associated protein tau
MAO	monoamine oxidase
Mb	megabase
MPTP	1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine
MSA	multiple system atrophy

mtDNA	mitochondrial DNA
P+	Parkinson plus
PD	Parkinson's disease
PEO	progressive external ophthalmoplegia
PET	positron emission tomography
POLG	mitochondrial DNA polymerase gamma
POLG1	gene encoding the catalytic subunit for POLG
PSP	progressive supranuclear palsy
SN	substantia nigra
SNCA	α-synuclein
SNP	single nucleotide polymorphism
SPECT	single photon emission computed tomography
VP	vascular parkinsonism

ABSTRACT

Slowness, stiffness, tremor, and postural instability are the cardinal symptoms of Parkinson's disease (PD). Since these symptoms are not specific to PD the diagnosis may be uncertain in early PD. Etiology and pathogenesis of PD remain unclear, and there is no neuroprotective treatment. Genetic findings are expected to reveal metabolic routes in PD pathogenesis and thereby eventually lead to therapeutic innovations.

The starting point of this thesis was to study the usefulness and accuracy of ^{123}I-β-CIT SPECT in the diagnosis of PD in a consecutive clinic-based material including various movement disorder patients. ^{123}I-β-CIT SPECT could distinguish PD from essential tremor, drug-induced parkinsonism, dystonia, psychogenic parkinsonism, and most cases from vascular parkinsonism, but β-CIT uptake in Parkinson plus syndromes and DLB was similar to that in PD. ^{123}I-β-CIT SPECT was 100% sensitive and specific in the diagnosis of PD in patients younger than 55 years but less specific in older patients, due to differential distribution of the above conditions in the younger and older age groups. ^{123}I-β-CIT SPECT correlated with symptoms and was able to detect a bilateral nigrostriatal defect in patients whose disease was still in unilateral stage. In addition to its use as a differential diagnostic aid, ^{123}I-β-CIT SPECT may also be used to detect PD early, even pre-symptomatically in at-risk individuals.

Subsequently we started a genetic project (II-V) to identify genetic risk factors for sporadic PD using candidate gene approach in a case-control setting, including 147 sporadic PD patients and 137 spouses as controls. ^{123}I-β-CIT SPECT was used as a part of the patient collection to the genetic studies.

We found no association between PD and *parkin* or *DJ-1*, genes underlying autosomal recessive parkinsonism. The functional Val158Met polymorphism, which affects the catalytic effect of COMT enzyme, and another coding polymorphism in *COMT* were not associated with PD in our patient material.

The *APOE* ε2/3/4 polymorphism modifies risk for Alzheimer's disease and prognosis of e.g. brain trauma. APOE promoter and enhancer region polymorphisms –219G/T and +113G/C, and *APOE* ε3 haplotypes, have also been shown to modify risk of

neuropathologically verified Alzheimer's disease but have not been reported in PD. In our study, no association was found between PD and the *APOE* ε2/3/4 polymorphism, –219G/T, +113G/C and the e3 haplotypes.

A genetic association was found with a polymorphic CAG-repeat in *POLG1,* the gene encoding the catalytic subunit of mitochondrial polymerase gamma, in both Finnish and North American PD patients. This CAG-repeat encodes a polyglutamine (polyQ) tract, the two most common lengths of which are 10Q (86-90%) and 11Q. In our Finnish material, the rarer non-10Q or non-11Q length variants (6Q-9Q, 12-14Q, 4R+9Q) were increased in frequency in patients compared to the spouse controls (10% vs. 3.5 %, p=0.003), and population controls (p=0.001). Therefore, we performed a replication study in a larger set of 652 North American PD patients and 292 controls. Non-10/11Q alleles were more common in US PD patients compared to the controls but the difference did not reach statistical significance (p=0.07). This larger data suggested that our original definition of variant length allele might need reconsideration. Most studies have defined 10Q as the only reference allele. Alleles other than 10Q, including also 11Q (non-10Q), were significantly more common in patients compared to the controls (17.3% vs. 12.3 %, p= 0.005). This significant association of non-10Q length variants and PD was seen also seen when compared to a larger set of 1541 literature controls (17.3 % vs. 12.8 %, p=0.00005). Our results suggest that *POLG1* polyQ alleles other than 10Q may predispose to PD.

INTRODUCTION

In 1817 a British physician, James Parkinson, first described a disease in his publication “An essay on the shaking palsy“, based on his observations of six patients: "Involuntary tremulous motion, with lessened muscular power, in parts not in action and even when supported; with a propensity to bend the trunk forward, and to pass from a walking to a running pace; the senses and intellects being uninjured." Later this disease was named Parkinson’s disease (PD) after him.

Tremor, slowness and poverty of movement (brady- and hypokinesia), and stiffness (rigidity) are three major motor symptoms of PD. Problems with controlling balance and posture follow. The patients’ motor ability declines in progressive course. Now we also know that “intellects being uninjured” is not true for around 30% of PD patients. Autonomic dysfunction, sleep disturbances, and depression are other possible non-motor symptoms.

The clinical picture of parkinsonism is not specific to PD, and therefore, the diagnosis may be difficult in the early phases of the disease despite strict clinical criteria[1, 2]. SPECT imaging of the dopaminergic system has been proposed to help in the differential diagnosis of PD.

PD is a neurodegenerative disease with an average age-of-onset around 60 years. Thus, with increasing life expectancy, the number of PD patients is expected to increase, even double during the next two decades [3]. Considering the remarkable PD-related disability, this is also an increasing challenge to society.

What causes PD remains unclear. PD typically occurs sporadically, and primarily environmental factors, such as pesticides or heavy metals, have been suspected to cause PD. Rare hereditary forms of PD exist. Mutations in eight genes have been so far identified as a cause of hereditary parkinsonism (OMIM, http://www.ncbi.nlm.nih.gov/entrez/dispomim.cgi?id=168600).

Genealogical and twin studies support the role of a genetic component also in the predisposition to the more common apparently sporadic PD[4]. The general view is that both environmental and genetic factors modify the predisposition to PD.

Genetic findings have revealed and are hoped to reveal metabolic routes in the so far poorly known pathogenesis of PD. Knowing the pathogenesis is needed to develop specific neuroprotective, maybe even preventive therapies. So far only symptomatic medication is available.

REVIEW OF THE LITERATURE

1. WHAT IS PARKINSON'S DISEASE?

1.1 Epidemiology of PD

PD is considered the second most common neurodegenerative disease after Alzheimer's disease[5]. PD is slightly more common in men [6]. Incidence rates reported for PD range from 8 to 18 per 100 000. PD might be less common in black and Asian people[5]. In industrialized countries the overall prevalence of PD is generally estimated about 0.3% in the entire population and 1.0% in population over 60 or 1.6% in population over 65 years of age [7]. PD is clearly an age-related disease: it is rare before age 50 years and prevalence increases with age, being up to 4% in age group 85-94 years [5, 8]. In Finland there are approximately 10 000 PD patients[9]. Along with increased life expectancy the number of PD patients has been estimated to double in the next 20 years[3].

1.2 Clinical manifestations and disease progression of PD

Parkinsonism is an entity of symptoms comprising of tremor, rigidity, hypokinesia, and postural instability. Idiopathic PD is the most common cause of parkinsonism. In PD, parkinsonism is often accompanied by a broader spectrum of other symptoms. The cardinal motor symptoms typically present initially asymmetrically. Tremor is usually resting tremor with a 4-6 Hz frequency beginning usually in either hand and spreading later to other limbs. Chin and lips may also be affected, and postural or action tremor may also occur.

Hypokinesia (smallness of movement) and bradykinesia (slowness) contribute to the slow shuffling gait, lost arm swing when walking, poor manual dexterity, small handwriting, hypomimia (loss of facial expression), monotonic silent speech, and dysphagia. Rigidity implies an increase in muscle tone, leading to a resistance to passive movement throughout the range of motion and

presents either as “cogwheeling” or smooth lead pipe type stiffness both in limb and later in axial muscles. Postural instability, falls and freezing usually manifest at later stage in PD patients.

Compared with healthy age-matched controls, PD patients have significantly more autonomic symptoms [10]. Autonomic system involvement manifests as cardiovascular (e.g. orthostatic hypotension), thermoregulatory, gastrointestinal (e.g. constipation, sialorrhea), urinary problems, and sexual dysfunction. Autonomic symptoms are present in virtually all PD patients at some stage of disease and contribute to the disease burden [11]. Older age, greater motor symptom severity and higher doses of dopaminergic medication are related with more autonomic problems [10].
Depressive disorders are estimated to affect up to 50% of PD patients and depression may be an early symptom of PD [12]. Psychotic symptoms such as visual hallucinations occur at later stages of PD, partly due to the disease process and partly due to dopaminergic medication, more often in patients with cognitive decline. Sleep disturbances, e.g. insomnia, daytime sleepiness, or REM sleep behavioural disorder, are common in PD patients [11].
Estimates of the prevalence of dementia or cognitive decline in PD have increased over the last years. Incidence of dementia in these patients is up to six times that in healthy people. Typically PD dementia affects about 30 % of patients and is characterized by impairment in attention, executive and visuo-spatial functions [13, 14]. Akinetic-rigid form of PD and older age are related to higher risk of dementia. In a recent longitudinal cohort study, 140 (60 %) of initial 233 PD patients developed dementia after a 12-year study period [15]. The cumulative incidence of dementia increases with age and duration of PD and, conditional on survival, increases to 80%-90% by age 90 years [15].

The clinical onset of PD is preceded by a pre-symptomatic phase of several years: nigral neuron loss is estimated to be 60% at the onset of symptoms [16, 17]. Before the onset of motor parkinsonism, some pre-motor symptoms may be present, including constipation, depression, sleep disturbances (especially REM

sleep behaviour disorder), and hypo-osmia [18], all logical considering the neuropathological progression of PD described in the next chapter.

The clinical course is progressive with highly variable rate of progression. On average, functional decline is more rapid and mortality higher in patients who predominantly present with non-tremor symptoms of PD, i.e. gait disturbance, akinetic-rigid syndrome, and cognitive decline [19, 20].

1.3 Neuropathology in PD

The classical pathological hallmarks of PD are the accumulation of intraneuronal cytoplasmic inclusions called Lewy bodies (LB) and Lewy neurites (thread-like protein aggregates in neuronal processes), and loss of the dopaminergic neurons of substantia nigra (SN) pars compacta projecting primarily to the putamen.
Lewy bodies were first seen and linked to Parkinson's disease ("paralysis agitans") in 1912 by the neurologist Frederic Lewy[21]. LB's are spherical eosinophilic cytoplasmic protein aggregates composed of α-synuclein and numerous other proteins: parkin, ubiquitin, synphilin and neurofilaments. The role of LB's in neuronal death is controversial. LB's are found in all affected brain regions but they are not specific to PD: they are also found in dementia with Lewy bodies (DLB), Alzheimer's disease, MSA, Hallervorden-Spatz disease and as an incidental pathological finding also in neurologically healthy people of advanced age[22, 23]. Recent neuropathological studies indicate that Alzheimer's disease, PD and DLB share many neuropathological features, indicating that at least partially shared neurodegenerative mechanisms operate in these disorders[24-26].

The loss of striatal dopaminergic neurons underlies the cardinal motor symptoms, but neurodegeneration extends beyond that. Neurodegeneration and LB formation are found in noradrenergic (locus coeruleus), serotonergic (raphe), and cholinergic (nucleus basalis of Meynert, dorsal motor nucleus of

vagus) systems, and in the cerebral cortex, olfactory bulb, and autonomic nervous system [17]. A recent staging procedure for the PD-associated brain LB pathology proposes that the process begins at two sites in the brain and spreads in a topographically predictable sequence in six stages. It has even been suggested that the disease may begin in the enteric nervous system and extend secondarily to the brain [27], a hypothesis still awaiting definite proof. It is unclear at which stage the autonomic nervous system is injured [28]. In stages 1 and 2, LB pathology is confined to the medulla oblongata, initially the dorsal motor nucleus of the glossopharyngeal and vagal nerves, and pontine tegmentum (including raphe nuclei, formatio reticularis) and olfactory bulb. Midbrain, particularly substantia nigra becomes involved in stage 3, and during stages 3-4 the disease is suggested to become clinically manifest [29]. In the final stages 5-6 the LB pathology spreads in the neocortex [29].

Table 1.
Clinico-pathological correlations of PD stages

Clinical phase	Pathology
Preclinical	Medulla oblongata, pontine tegmentum, olfactory bulb, autonomic? (Stages1-2)
Early- moderate PD	Medulla oblongata, pontine tegmentum, olfactory bulb, autonomic?, substantia nigra (Stages 3-4)
Severe PD, PDD	Medulla oblongata, pontine tegmentum, olfactory bulb, autonomic, substantia nigra, cortex (Stages 5-6)

Simplified from [28, 30, 31].
PDD= PD with dementia

1.3.1 Selective neuronal vulnerability

An important question in elucidating the pathogenesis of a disease is the selective vulnerability of specific cell types. Neuronal vulnerability or resistance may be affected by cell size and localization, metabolism of disease-specific proteins and a repertoire of signal transduction pathways and stress resistance mechanisms [32]. Because dysfunction and death of neurons affect adversely both the pre- and post-synaptic neurons they communicate with, the patterns of progression of neuronal degeneration are often domino-like [32].

In PD the degenerating cell population is mostly, but not exclusively as discussed above, the dopaminergic nigrostriatal neurons. For PD, *preferential*, instead of selective, neuronal vulnerability is a better expression.

Even though the degeneration of the dopaminergic neurons of the SN pars compacta is the most prominent feature in PD, and about 60 % of them are already lost at the time of diagnosis [16], not all brain dopaminergic neurons are equally affected in PD: in post-mortem PD brains 40-60% of the mesolimbic /mesocortical projections from the ventral tegmental area are lost [33, 34], but the dopaminergic neurons from nucleus arcuatus of the hypothalamus that regulate anterior pituitary secretion are not lost [35].

2. DIAGNOSIS OF PD

2.1 Clinical diagnosis

Diagnosing PD may be challenging in the early phases of PD because of the variability of the PD phenotype and the significant overlap between other parkinsonian syndromes. No clinical sign or symptom is specific to PD.
The diagnosis of PD is based on findings of parkinsonism on neurological examination and exclusion of other causes for parkinsonism using neurological examination, medical history, trial of antiparkinsonian drugs, and follow-up. Neuroimaging, neurophysiological studies or laboratory tests are usually not needed. The most commonly used diagnostic criteria for PD are the United Kingdom Parkinson's Disease Society Brain Bank clinical diagnostic criteria presented in table 2 [1].

Post-mortem studies have found error rates of PD diagnosis as high as 24% [1]. In another clinicopathological study, Rajput et al found that clinical diagnosis within 5 years of disease onset was correct in 65% of cases. After a mean duration of 12 years, the final diagnosis of PD by the clinician was confirmed at autopsy in 76% of cases [36]. With strict criteria the accuracy of a clinical

diagnosis of the disease can be improved significantly, but up to 10% of patients diagnosed with PD in life will still have to be reclassified at autopsy [2].

Table 2. UK PD Society Brain Bank criteria for idiopathic PD (modified from [1])

INCLUSION CRITERIA	SUPPORTIVE CRITERIA	EXCLUSION CRITERIA
Hypokinesia	Asymmetric onset	Repeated head trauma
AND at least 1 of the following	Progression of the disease	Repeated stroke
Rigidity	Persistent asymmetry	History of encephalitis
4-6 Hz resting tremor	Resting tremor	Ocylogyric crises
Postural instability	Good response to L-dopa	Neuroleptic therapy at onset
	L-dopa-induced dyskinesia	Persistent remission
	Persistent (≥5 years) response to L-dopa	Purely unilateral symptoms 3 years after onset
	Long clinical course (≥10 years)	More than 1 relative with parkinsonism *
		Supranuclear gaze palsy
		Cerebellar signs
		Early severe autonomic dysfunction
		Early severe dementia
		Babinski sign
		Brain tumor or hydrocephalus
		Lack of response to L-dopa
		Exposure to MPTP

* hereditary parkinsonian syndromes are not classified as idiopathic PD here

2.2 The most important differential diagnoses

Symptoms of parkinsonism are also caused by other mechanisms than PD, with or without nigrostriatal pathology. Early falls, poor response to L-dopa, rapid progression of symptoms, disturbance of eye movements, problems with swallowing or speech, pyramidal signs and early autonomic involvement indicate diagnosis other than idiopathic PD [37].

Structural imaging of the brain with CT or MRI is normal in PD, but may have value in excluding other causes such as vascular parkinsonism (VP), tumors, normal pressure hydrocephalus (NPH). MRI may also be helpful in differentiating PD from Parkinson plus (P+) syndromes as in these several structural MRI changes have been described: putaminal, pontine, and middle cerebral peduncle atrophy and T2 signal changes in the basal ganglia and

brainstem in multisystem atrophy (MSA), midbrain atrophy in progressive supranuclear palsy (PSP), and asymmetric frontoparietal atrophy in corticobasal degeneration (CBD) [38, 39]. Clinical neurophysiology is of little value in diagnosing parkinsonism in routine clinical practice [40]. Laboratory testing is also of little value, except in excluding Wilson's disease, or SCA-2, SCA-3 or SCA-17 in rare cases [40].

In P+ syndromes parkinsonism occurs with other features.
PSP, neuropathologically a tauopathy, is the most common P+ syndrome. In PSP loss of vertical eye movements and cognitive decline accompany akinetic-rigid type parkinsonism and early postural instability [41]. CBD is another tauopathy, the special clinical features of which are marked persistently asymmetric rigidity of the limbs, especially upper limb, "alien limb" sign, dysarthria, dysphasia, sensory symptoms and apraxia [42]. Multiple system atrophy (MSA) was earlier considered as three different diseases: Shy-Drager syndrome, striatonigral degeneration, and sporadic olivopontocerebellar atrophy. At present, they are categorized as MSA-P (patients with predominantly parkinsonian features) or MSA-C (patients with predominantly cerebellar ataxia), depending on the predominant feature when the patient is evaluated. In MSA, marked autonomic dysfunction presents early particularly as orthostatic hypotension and collapses; in PD autonomic symptoms usually occur later. Also postural instability shows up earlier in MSA than in PD. Parkinsonism is usually of akinetic-rigid type, and response to L-dopa is poor [39]. In dementia with Lewy Bodies (DLB) early progressive dementia, cognitive fluctuation, hallucinations, marked sensitivity to neuroleptics combine with parkinsonian symptoms [43]. The neuropathological features of DLB and PD overlap: both have LB's but their distribution is different [31].

False PD diagnoses are most commonly essential tremor (ET) [40], which is characterized by presence of bilateral, largely symmetrical, postural or kinetic tremor that affects the hands and forearms and is visible and persistent. Bradykinesia, rigidity, and postural instability are not part of ET [44]. Difficult

diagnostic situations arise when an otherwise typical case of ET is associated with rest tremor (10% of cases) or when the tremor is unilateral [45, 46]. The presence of family history and relief by alcohol strongly favour the diagnosis of ET [44].

Secondary parkinsonism may result from drugs, cerebrovascular disease, trauma, infections (post-encephalitic parkinsonism) and toxins (e.g. manganese, carbon monoxide, MPTP). Drug-induced parkinsonism (DIP) is usually caused by antipsychotics, but also other drugs, e.g. metoclopramide, calcium channel blockers, lithium, and natriumvalproate may provoke parkinsonian symptoms. Tremor is less common than hypokinesia and rigidity, and the symptoms are typically symmetrical. DIP is in most cases reversible after discontinuation of the causative drug [47]. Vascular parkinsonism (VP) is a controversial entity. It is often characterised by widespread bilateral deep lacunar white matter infarcts and typically causes a parkinsonian gait disorder with freezing and wide-based small stepped gait (lower body parkinsonism). Onset is usually gradual, but striatal or SN infarcts can give rise to acute onset of contralateral parkinsonism [48, 49].

2.3 SPECT and PET imaging of the dopaminergic system in PD

As described above, the diagnosis of PD is usually clinical, but ancillary methods may be considered for some selected problematic cases.

Dopamine transporters (DAT) are dopamine re-uptake proteins on the presynaptic terminals of dopaminergic neurons [50, 51]. Measuring DAT density using DAT ligands and single-photon emission computed tomography (SPECT) or positron emission tomography (PET) provides *in vivo* information on the integrity of these presynaptic terminals. In PD, as the number of dopaminergic neurons is estimated to be reduced to 30-40% at the time of diagnosis [16], DAT density is reduced. In PD, the number of DATs per neuron may also be lower

than normal because of the attempt to increase the amount of synaptic dopamine by means of reducing the re-uptake [52].

^{123}I-β-CIT (2β-carbomethoxy-3β-(4-iodophenyl)tropane) is a cocaine derivative and a radioligand which binds to DATs and serotonin transporters (SERT). Striatal β-CIT binding is mainly associated with DAT, whereas binding in the hypothalamus and midbrain is mainly associated with SERT [53]. The binding kinetics of β-CIT is different for SERT and DAT: uptake in the striatum is slow, reaching a maximum approximately 16 h after tracer injection, followed by stable binding for up to 24 h after injection in humans. Peak uptake to the hypothalamus and midbrain is observed approximately 4h after injection. Thus, ^{123}I-β-CIT SPECT shows substantial striatal DAT specificity at approximately 24 hours after injection [54].
Other available DAT SPECT *tracers* are FP-CIT (DATSCAN, ioflupane) and ^{99m}Tc-TRODAT-1 with different binding kinetics [55, 56]. The faster kinetics of ^{123}I-FP-CIT allows adequate acquisition as early as 3 h following injection [57].
18F-Fluorodopa (F-dopa) PET also correlates with the number of nigrostriatal neurons. Its uptake expresses the activity of dopa-decarboxylase and dopamine storage capacity in the striatal nerve terminals of the dopaminergic neurons [58]. Limitation of F-dopa is that dopa-decarboxylase is up-regulated early in the disease process. This may lead to underestimation of dopaminergic neuron loss by F-dopa PET. Therefore, DAT binding tracer may provide more accurate estimate of nigrostriatal degeneration [59, 60]. Comparative studies show that results of DAT imaging with SPECT correlate well with PET results [61, 62]. SPECT is more widely available than PET and less expensive.

In contrast to DATs, dopamine receptors are mainly located on the striatal gabaergic neurons projecting to the globus pallidus. ^{11}C-Raclopride-PET or ^{123}I-Epidepride- or ^{123}I-Iodobenzamide (IBZM) SPECT may be used for *postsynaptic dopamine receptor imaging.* Dopamine receptor levels are not reduced in PD; in early PD the level of dopamine receptors may even be upregulated [63, 64].

2.3.1 ^{123}I-β-CIT SPECT in PD and differential diagnoses

DAT density measured with ^{123}I-β-CIT uptake is significantly lower in PD compared to healthy controls [65-67]. Reduction of β-CIT uptake also correlates with symptom severity [66, 68]. β -CIT uptake decreases also along normal ageing in healthy controls approximately 0.8% in a year [69], or 6-10% in a decade [53, 70,71].

In patients with purely unilateral symptoms (Hoehn-Yahr stage I), the reduction of β-CIT uptake is more evident in the contralateral striatum but striatal DAT density is also reduced in the ipsilateral striatum [72, 73]. Reduced DAT density has also been found in patients with REM-sleep behavioural disorder, which is a possible pre-motor symptom of PD [74]. These findings indicate that β-CIT SPECT can also detect preclinical PD.

In approximately 10% of patients with clinical suspicion of PD, a normal DAT SPECT has been reported. It has been proposed that most of these SWEDDs (Scans Without Evidence of Dopaminergic Deficit) patients have dystonic tremor [75].

Striatal ^{123}I- β-CIT uptake is also reduced in MSA, including the cerebellar type, and PSP [76-79]. Some studies have suggested that PD might be differentiated from these P+ syndromes using β-CIT SPECT, but most have failed to find a significant difference in striatal ^{123}I –β-CIT uptake between PD and MSA or PSP. In PD the reduction of β-CIT uptake is most pronounced in the putamina whereas the caudate nuclei are relatively spared, in accordance with findings of neuropathological studies [80]. In MSA and PSP the striatum might be more uniformly affected compared to PD [77, 81-83]. Also asymmetry of the reduction of β-CIT uptake is more typical in PD compared to MSA or PSP, but this difference has not reached statistical difference in most studies [78, 79, 82, 84].

In addition to PSP and MSA, reduced striatal β-CIT uptake has been reported in dementia with Lewy bodies [85], Wilson's disease [86], spino-cerebellar ataxia type 2 (SCA2) [87] and corticobasal degeneration [78]. In contrast, striatal DAT density as visualized by β-CIT SPECT is normal in essential tremor [88], dopa-responsive dystonia [89, 90] and cervical dystonia [91].

Gerschlager et al suggested vascular parkinsonism (VP) could be differentiated from PD using β-CIT SPECT [92]. In their study, DAT density of the VP patients was within normal range, but the results of consequent FP-CIT studies on DAT density in VP patients have been conflicting [93, 94]

β-CIT SPECT in drug-induced parkinsonism (DIP) has not been reported. Mostly normal FP-CIT uptake indices in small groups of patients have been reported, but in some patients with irreversible DIP diminished DAT density has been found, suggesting possible exacerbation of underlying neurodegenerative parkinsonism in these patients [95-97]. In patients with tardive dyskinesia, FP-CIT uptake is normal [98].

Results of other DAT SPECT ligands are parallel to those of β-CIT in PD and differential diagnoses.

2.3.2 SPECT and PET imaging in genetic forms of parkinsonism

Patients with *parkin* (PARK2)-related parkinsonism seem to have severe and more symmetric reduction in striatal DAT density than in PD, as measured with FP-CIT SPECT [99]. In *PINK1*-related disease, F-dopa PET and DAT SPECT findings are similar to those of PD [100, 101]. However, both dopaminergic neuron loss and normal-range DAT densities have been reported in heterozygous *PINK1* mutation carriers [100, 101]. In patients with DJ-1 mutation, DAT density is in the range of idiopathic PD [102].

F-dopa PET in patients with AD parkinsonism caused by mutations in *SNCA* (PARK1) or *LRRK2* (PARK8) demostrates a similar degree and pattern of

nigrostriatal degeneration as in idiopathic PD [103, 104]. Thus, the nigrostriatal degeneration in these genetic forms of parkinsonism, seems to be similar to idiopathic PD by SPECT or PET imaging.

3. TREATMENT OF PD

Treatment of PD consists of medication, exercise, and in some rare cases surgery. So far, drug treatment of PD is symptomatic dopamine (DA) replacement therapy. *Levodopa* (L-dopa), an immediate precursor of DA, has been used to relieve PD symptoms since the late 1960's [105] and it is still the most important and most efficient drug in PD. Long-term treatment of L-dopa results in the development of motor complications after five years of L-dopa treatment in half of the patients or more [106]. The effect of L-dopa becomes shorter (wearing off) and dyskinesias occur usually when the plasma levels of L-dopa are highest (peak-dose dyskinesia). Co-administration of L-dopa with peripheral dopa-decarboxylase inhibitors (benseratzide or carbidopa) diminishes peripheral formation of DA and peripheral side effects of L-dopa, and increases the amount of L-dopa entering the brain where it is converted to DA in the dopaminergic neurons. *COMT inhibitors* (entacapone, tolcapone) also increase L-dopa availability in the striatum by inhibiting its COMT-dependent conversion into inactive metabolites. COMT inhibitors are only used with L-dopa and may allow reduction of L-dopa dose and prolong the L-dopa dosing interval [107, 108].

Dopamine agonists mimic the actions of DA in the striatum and stimulate DA receptors directly. DA agonists are effective as monotherapy in early PD, and with L-dopa in more advanced disease [109]. However, agonists are not as effective as L-dopa, especially in late-stage disease, and L-dopa is eventually needed in most patients. [110] Early use of DA agonists as monotherapy or as L-dopa add-on may delay motor complications in comparison to L-dopa monotherapy [106, 111].

MAO-B inhibitors (selegiline and rasagiline) reduce catabolism of striatal DA and thereby increase DA levels in SN and basal ganglia [112]. Selegiline has antiparkinsonian efficacy as monotherapy in early PD and may delay the need for L-dopa [113, 114], but its effect is only mild to modest. Selegiline and rasagiline can extend the effect of L-dopa and improve symptoms in L-dopa-treated patients with fluctuations [115, 116]. *Anticholinergics* were the first and only drugs with therapeutic efficacy for PD from the late 1940's until late 1960's. Anticholinergics may be useful in tremor-predominant PD but their efficacy is not comparable to L-dopa [110] and cognitive adverse effects limit their use particularly in older patients [117].
Antiparkinsonian properties of a*mantadine*, originally introduced as treatment and prophylaxis of influenza A, were reported late in the 1960's [118]. Amantadine acts as an NMDA antagonist, can increase dopamine secretion and inhibit its re-uptake, and has some anticholinergic effects [119, 120]. Amantadine's therapeutic effect is only mild [121]. It may relieve dyskinesias in L-dopa-treated fluctuating patients [122].

Deep brain stimulation (DBS) of the subthalamic nucleus, globus pallidus, or thalamus may be used for some cases with severe fluctuations or intolerance to drug therapy and can relieve a wide range of symptoms [123]. Thalamic DBS may be an option for patients with severe tremor [124].

Stem cell transplantation has only been in experimental use. Uncontrolled open-label trials of fetal dopaminergic grafts on advanced PD patients have reported clinically meaningful improvement [125-127], but a placebo-controlled double-blind trial found no significant overall treatment effect, and a major problem has been the appearance of persisting dyskinesia in most patients with transplants [128]. Post-mortem studies have revealed another problem with this method: the disease process seems to spread to the transplant [129, 130].

Coenzyme Q10 (ubiquinone) seemed to delay functional decline of PD patients in a small preliminary study [131] but no established neuroprotective therapy is currently available for PD.

4. ETIOLOGY OF PD

4.1 Environmental risk factors

In the early 80's it was discovered that several people developed parkinsonism after injections of drugs contaminated with 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) [132, 133]. This discovery suggested that environmental toxins may be related to risk of PD.

Smoking cigarettes and drinking coffee have been consistently shown to protect from PD [134-136]. Fairly consistent evidence suggests association between pesticide exposure and PD risk [137, 138]. Increased PD risk has also been connected to rural living and well water use [139] as well as welding and exposure to heavy metals such as lead, manganese, iron, and copper [137], the evidence for these associations still being inconclusive [5, 140]. Repeated head trauma may increase risk of PD [141].

Results considering nutrients in PD risk are incoherent. Polyunsaturated fatty acids have been suggested to decrease and consumption of dairy products increase PD risk [5]. Whether cholesterol has an effect on PD risk, and to which direction, needs further studies: *High* cholesterol levels have been found to increase susceptibility to PD in a recent Finnish study [142], whereas *low* cholesterol, especially low LDL, in another study [143].

4.2 Genetic factors

4.2.1 Genetic epidemiology

Most PD cases occur sporadically, and PD was long thought to be of purely environmental origin. In recent years increasing evidence has been found for the significance of a genetic component in the etiology of PD based on pedigree analyses and twin studies. Earlier twin studies relied on clinical criteria and found low concordance levels, which lead to the interpretation that genes have little or no effect in PD susceptibility [144, 145]. However, a large study of male twins reported a significant effect of genetic factors in patients with age at PD onset less than 50 years [146]. An Icelandic genealogical study found markedly increased risk of PD in relatives of patients with late onset PD, and thus provided evidence for a contribution of genes also in the more common sporadic late-onset form of this disease [4]. Another twin study, in which F-DOPA PET was used to detect subclinical PD, found significantly higher concordance for subclinical PD in monozygotic compared to dizygotic twins (55% vs. 18%), which further confirms the role of genes in PD [147]. Since late 1990's, rare monogenic forms of PD have been recognized [148]. Only in late 1990s a consensus emerged that idiopathic sporadic PD seems to result from interplay of genetic and environmental factors.

4.2.2 Genes or loci of familial PD

To date, eight genes and five genetic loci for familial Mendelian inherited PD are known (OMIM 168600). These 13 PARK loci are summarized in table 3.

Table 3. The genetic loci of familial parkinsonism. (OMIM #168600)

LOCUS	MAP POSITION	GENE	INHERITANCE	ONSET	POPULATION	CLINICAL AND PATHOLOGICAL FEATURES	Ref.
PARK1	4q21	Alphasynuclein (*SNCA*)	AD	40s	Italian, Greek, German	LB+ nigral degeneration, typical and atypical parkinsonism, dementia	149
PARK2	6q25.2-q27	*Parkin*	AR	20-40	Japanese, Spanish, Italian, Brazilian	Early (even juvenile) onset Classic symptoms of parkinsonism+ foot dystonia, marked sleep benefit, hyperreflexia in some Different pathology:no LB's	150
PARK3	2p13	unknown	AD	60s	European	LB+ nigral degeneration, some have plaques and tangles	151
PARK4	4q21	*SNCA*, duplication or triplication	AD	30s	English-German Swedish-American	Early age at onset, early weight loss, rapidly progressive l-dopa-responsive parkinsonism, dementia, LB+ degeneration	152
PARK5	4p14	*UCHL1*	AD	50	German	Clinically typical parkinsonism with response to l-dopa. Pathology not reported	153
PARK6	1p36	*PINK1*	AR	30-40	Asian, Italian	Most patients have features similar to those of idiopathic PD, a subset demonstrate features similar to those of PARK2. Slow progression. Pathology not reported	154
PARK7	1p36	*DJ-1*	AR	30-40	Dutch, Italian	Clinically typical + psychiatric disturbances. Pathology not reported	155
PARK8	12q12	*LRRK2*, dardarin	AD	60	Japanese, Basque,English, Ashkenazi Jewish German Canadian	Most common known cause of AD parkinsonism. Clinically mostly typical. Pathology variable, LB's in most, tau in some	156
PARK 9	1p36	*ATP13A2*	AR	20-40	South-American, Jordanian	Kufor-Rakeb syndrome, early onset parkinsonism with dementia, supranuclear upgaze paresis, pallidopyramidal degeneration	157
PARK10	1p32	unknown	AD?	60	Icelandic	Typical, late-onset Pathology not reported	158
PARK11	2q36-q37	unknown	AD?	Late?	North American	Clinically typical, pathology not reported	159
PARK12	Xq21-25	unknown	X-linked	Late?	North American	Clinically typical, pathology not reported	160
PARK13	2p12	*HTRA2*	AD?	49-77	German	Typical parkinsonism, response to l-dopa. Pathology not published	161

4.2.3 Genes in sporadic PD

4.2.3.1 Approaches in genetic studies of sporadic PD

In Mendelian traits a single mutated disease gene causes the disease in subjects carrying one (dominant) or two (recessive) copies.
In multifactorial diseases, such as PD, the particular disease gene does not directly cause the disease but confers susceptibility to it. Under particular environmental conditions subjects with predisposing alleles may develop the disease. A straightforward common disease-common variant (CD-CV) hypothesis suggests there may exist common variants that contribute to genetic risk for common diseases [162, 163]. A prototypic example supporting this still debatable hypothesis is the contribution of APOE ε4 allele in Alzheimer's disease and cardiovascular disorders [164, 165].

Genetic association studies try to identify disease genes by finding association between a genetic marker and the disease phenotype. A genetic marker is associated with the disease if the frequency of the marker allele or genotype differs significantly between affected and unaffected individuals. Association studies are based on linkage disequilibrium (LD) [166-168]. This means indirect detection of the disease allele with an adjacent genetic marker ("guilt by neighbourhood"). Hence, association between the disease and the marker allele does not imply causal effect of this marker allele but reflects its LD with the disease allele.
The magnitude of LD between the marker allele and disease allele is determined by at least five factors [167]. 1.Recombination frequency between the two alleles, which roughly correlates with the distance of the alleles. 2. Age of the mutation creating the younger allele. The older the mutation event creating the younger allele, the more time there has been for recombination to equilibriate the association. 3.Single origin vs. multiple origins of the alleles. If both alleles have been created only once, LD is stronger as compared to the situation in which one or both alleles have been created multiple times by mutation. 4. The frequency of the allele adjacent to the younger new mutation. If a new mutation occurs in the neighbourhood of a high frequency allele, LD is

lower than when it is paired with a rare allele. A high frequency allele is a less specific marker of a new mutation than a rare allele occurring in combination with the new mutation. 5.Characteristics of the study population. Features that tend to increase LD are recent founding of the population, low number of founders, isolation of the population, bottlenecks during the population history (famines, wars), genetic drift [169]. Non-random mating and inbreeding also tend to increase LD of the population.

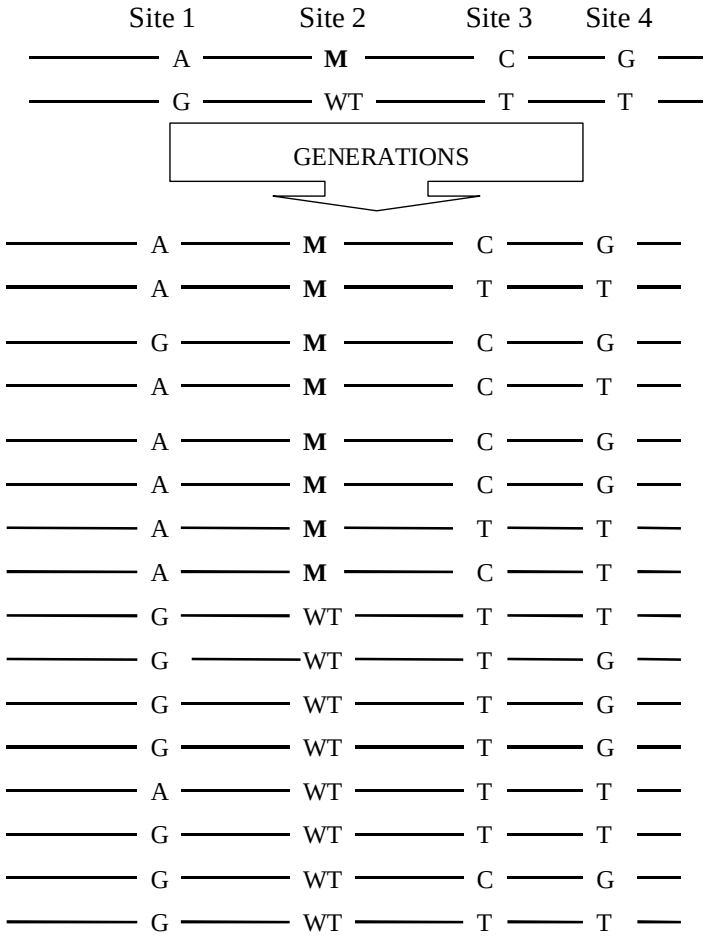

Figure 1. The idea of association studies utilizing LD. A mutation (**M**) occurs on an ancient chromosome surrounded by 3 SNP's with alleles A, C, and G, whereas in wild type (WT) the alleles of the corresponding SNP's are G, T, and T. Through generations recombinations happen, but statistical connection of the closest alleles and the mutation remains detectable. Here 7/8 (88%) of A alleles on site 1, 6/7 (86%) of C alleles in site 3 mark the mutation indicating LD. Site 4 is no more in apparent LD with the mutation after multiple generations: G occurs equally frequently (50%) in combination with the mutation and the wild-type allele.

The physical dimension of LD varies in different genomic regions and between populations. A whole-genome average estimate of 60 kb for practically meaningful LD (|D'|>0.5) for disease association studies has been suggested [170].

Genetic association studies have traditionally focused on candidate genes based on hypotheses on the disease pathogenesis. Candidate gene studies have been widely used to identify susceptibility genes in many multifactorial diseases, including PD. Genome-wide association (GWA) studies use high-throughput genotyping technology to assess up to hundreds of thousands of SNP's across the genome and relate them to disease phenotype. Generally the associations found by GWA may be considered preliminary and need to be confirmed by other studies. When hundreds of thousands markers are tested the number of comparisons is so high that p-values of 10^{-5} are expected to occur several times by chance alone. Replication is here the proof rather than a single low p-value.

4.2.3.2 Problems in interpreting the data

Association of PD with more than 200 candidate genes has been studied (Appendix). Association of some genes and PD has been found repeatedly and some are clearly negative, but the role of many of these genes is still inconclusive. For instance, what to think of the role of a gene in PD susceptibility if there are three studies with positive (association with PD) and six with negative results? When interpreting the published data, one has to be aware of the many potential sources of error in genetic association studies.

- Positive findings tend to get published more readily than negative ones (publication bias).
- Population substructures affect case-control association studies as cases and controls may derive from distinct subpopulations (population stratification bias). Family-based association studies have been developed to overcome this problem [171], but these are not easily applicable to PD due to the high age of the patients (parents often deceased).
- The genetic association may be restricted to certain population(s) only (genetic heterogeneity).
- The genetic methods may not be comparable: Some studies have directly searched for mutations and some have tested association of

common variation indirectly, via linkage disequilibrium, with PD. Moreover, in some studies only one or few polymorphisms per gene have been studied, which may be too little to for the evaluation of a gene's role in disease susceptibility. More comprehensive analyses with numerous markers per gene and haplotype analyses possess higher genomic information content. Analysis of a polymorphism that has functional consequence on protein function is usually more "informative" than analysis of a non-functional marker.

- Different size patient materials have been used, some of which possibly have insufficient power.
- Patient selection criteria may not be identical (phenotypic heterogeneity).

Meta-analysis is one means of reducing the noise from conflicting reports. However, albeit helpful in interpreting the exuberant data, meta-analyses also face some problems: publication bias tends to magnify positive findings. On the other hand, the use of large combined material in the meta-analysis may lead to loss of population-specific associations. Another problem may be that search engines may omit some studies that have not used the most commonly searched words and these studies might thus be left out of meta-analyses.

4.2.3.3 Expansion of data

A PubMed search in April 2008 with key words "sporadic", "Parkinson's disease", and "gene", limited to humans and English language, yielded 432 results. Limited to publication years 1990-1994, this search yielded only seven results. A search limited to the next five years (1995-1999) found 74 results, 162 in the five years thereafter, and 189 in the last four years. A closer look at these search results revealed that 3 out of 7, 25 out of 74, 64 out of 162, and 104 out of 189 were genetic candidate gene association studies on sporadic PD. Surprisingly many other published studies, not found in the PubMed search although published in well-known peer-reviewed journals, were found via a database for Parkinson's disease genetic association studies in www.PDGene.org by Massachusetts General Hospital / Harvard Medical School

and Michael J. Fox Foundation. Thus, the search results by PubMed are far from inclusive, but do indicate the rapid growth of articles in this field.

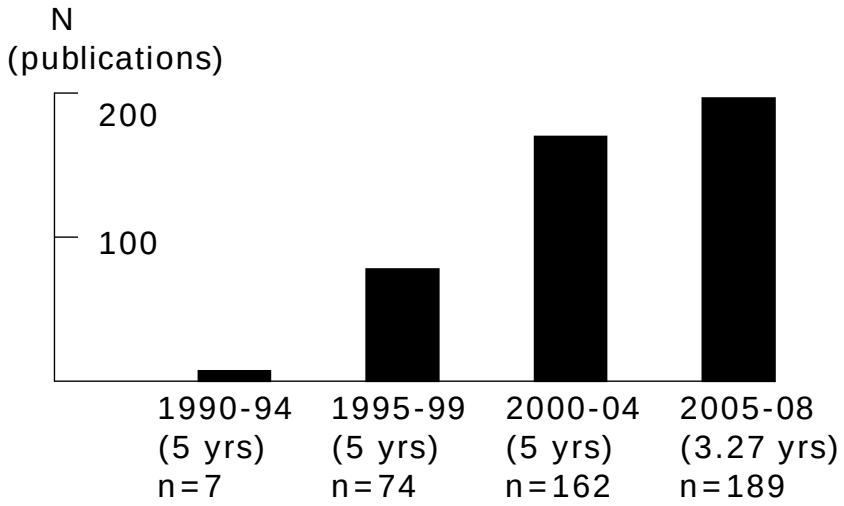

Figure 2. Growth in the number of publications on the genetics of sporadic PD. PubMed search of original articles in peer-reviewed journals using English language (keywords “sporadic”, “Parkinson’s disease”, “gene”).

4.2.3.4 Selected candidate genes analyzed in sporadic PD

The genes of monogenic parkinsonism (table 3) are potentially interesting candidate genes also in the etiology of sporadic PD due to the clinical and neuropathological similarity of the diseases. Of these genes, *SNCA* has shown allelic association with sporadic PD in several studies [172-177] but the mutations of monogenic PD or multiplications are rare in sporadic patients [178-183]. *LRRK2* mutations, the most common known cause of AD parkinsonism so far, are also found in apparently sporadic PD patients in several populations. Most common of the *LRRK2* mutations is a low penetrance mutation G2019S, which is most common in North African Arabs (as high as 41% in sporadic patients) and Ashkenazi Jews (~29% familial and ~13% sporadic patients) [184, 185]. It is also present in relatively high frequency in Southern Europe such as Italy, Spain and Portugal (5-18% in familial and 1-6% in sporadic patients) [186-188] whereas in Northern European patients (Scandinavian, Russian, Polish, British) it is much less common (0-1.6% in sporadic patients) [189-193]. The carriers of G2019S share an ancestral haplotype [186] suggesting a common founder, which explains the remarkable interpopulation variation in frequency.

In addition, association of sporadic PD and genes involved in other neurodegenerative disorders, dopaminergic transmission or dopamine metabolism, xenobiotic metabolism, mitochondria, and immune response has been extensively studied. Several associations have been reported but few associations have been confirmed in subsequent studies. Of the 265 genes (Appendix) studied in association to PD susceptibility only the most interesting ones are presented here and in table 4. *Parkin, DJ-1, APOE, COMT* and *POLG* are discussed in more detail because they are included in the studies of this thesis.

Table 4. Interesting candidate genes studied in sporadic PD

Gene	Association or mutation analyses		Comments	Refs
	Mostly positive	Conflicting		
Genes of monogenic PD				
SNCA	X		Allelic association with non-coding markers in many studies. Missense or nonsense mutations and multiplications rare	172, 174, 176, 177
LRRK2	X		Low penetrance mutation G2019S in 0-41% of apparently sporadic patients	185, 194-198
DJ-1		X	No association with sporadic PD	199, 200
Parkin		X	Mutations present in juvenile and early onset patients. Possible associations of coding SNP's and sporadic PD.	201-207
PINK1		X	Heterozygous mutations, in some reports, slightly enriched in sporadic patients.	208-210
UCHL1		X	Some studies have suggested S18Y protective	211-213
Other genes involved in neurodegeneration				
APOE		X	Reports of both ε2 and ε 4 in increased PD risk. Associations with PD dementia or onset age suggested	214-220
MAPT (*tau*)	X		OR 1.3-1.4 for the common H1 haplotype homozygocity	221
Glucocerebrosidase(GBA)	X		The gene of Gaucher's disease,	222-224
POLG1		X	Two small negative studies on *POLG1* in sporadic PD	225, 226
Genes involved in DA metabolism or dopaminergic transmission				
COMT		X	Val / Met polymorphisms effect to PD medication also studied (no effect)	227-231
DAT		X		232-235
Monoamine oxidase B (MAO-B)		X		236-239
Genes involved in the metabolism of toxins				
CYP2D6		X	Poor metabolizer genotype suggested to increase PD risk in few studies, possible pesticide interaction	240-243
Alcohol dehydrogenase 1c (ADH1c)	X	X	Only one study (positive)	244
N-acetyltransferase (NAT2)		X	Slow acetylator genotype suspected	245-248
Glutathione-s-transferases (e.g.GSTP1, GSTPM1)		X	Possible gene interaction with herbi- / pesticides	249-252
Paraoxonase 1(PON1)		X		253, 254

4.2.3.4.1 *Parkin*

Parkin protein functions in the cellular ubiquitination/protein degradation pathway as an E3 ubiquitin ligase. Loss-of-function mutations of *parkin* are suggested to lead to accumulation of non-ubiquitinated substrates, detrimental to the dopaminergic neurons. Patients with mutations in the *parkin* gene generally do not show ubiquitin-positive Lewy bodies, which is probably explained by the failure of ubiquitination [255].

Parkin gene is located on chr 6q25 with 12 exons spanning 1.3Mb of genomic DNA. *Parkin* mutations were first described in Japanese families with juvenile-onset autosomal recessive parkinsonism [150]. Since then, PD-associated *parkin*-mutations have been described in all exons, and include point mutations, small insertions/deletions and larger deletions, and exon duplications and triplications [256]. *Parkin* mutations have turned out to be a common cause of early-onset parkinsonism, especially in patients with evidence for recessive inheritance [257, 258]. *Parkin* mutations are also responsible for majority of sporadic cases with very early (<20y) onset and are common (25%) in patients with onset at 20-35 years of age [258, 259]. Some studies have found heterozygous *parkin* mutations in early- and late-onset patients but not in controls, suggesting also heterozygous *parkin* mutations may increase susceptibility to PD [260, 261]. However, heterozygous *parkin* point mutations were as common in cases as in controls in another study [262]. Therefore, the role of heterozygous parkin mutations remains a matter of debate. Some coding polymorphisms of *parkin* have been suggested to associate with sporadic PD in Japanese as well as European populations [201, 203, 207].

Early onset, diurnal fluctuations of symptoms, benefit from sleep, foot dystonia, hyperreflexia, and good response to l-dopa but early susceptibility to levodopa-induced dyskinesias are typical features of patients with *parkin* mutations. Lewy bodies are typically not found [259, 263]. Nevertheless, the phenotypical spectrum may be broader since a phenotype indistinguishable

from typical idiopathic PD has been reported in a pedigree from South Tyrol with compound heterozygous *parkin* deletions [264].

4.2.3.4.2 *DJ-1*

DJ-1 protein has been ascribed various functions but the most relevant in terms of the pathogenesis of PD is its potential role in oxidative stress response, either as redox sensor or antioxidant protein, and relation to mitochondrial function. In the presence of oxidative stress, wildtype DJ-1 translocates to outer mitochondrial membrane and is supposed to be involved in neuroprotection [265, 266]. DJ-1 expression in the brain is ubiquitous, with higher levels of the transcript in the caudate nucleus, thalamus, substantia nigra, and hippocampus [155].

DJ-1 gene on chr 1p36 contains 8 exons spanning 24 kb. The first two exons are non-coding and alternatively spliced in the mRNA. *DJ-1* mutations include missense, truncating, splice site mutations and large deletions, and are the rarest among the known genetic causes of autosomal recessive parkinsonism, being present in about 1-2% of early-onset cases [102, 155, 199, 256, 267]. DJ-1 mutations contribute rarely to the development of sporadic PD [199].

4.2.3.4.3 Apolipoprotein E (APOE)

APOE (Chr 19q13) gene has three common alleles designated ε2, ε3 and ε4 determined by amino acid substitutions arginine or cysteine in positions 112 and 158 of the APOE protein. ε4 allele is a risk factor for Alzheimer's disease [164, 268] whereas ε2 is protective and ε3 has been considered "neutral". APOE ε2/3/4 polymorphism has also been shown to affect prognosis after brain haemorrhage or trauma [269, 270]. Also ε3 haplotypes have been reported to be different and affect risk of neuropathologically verified Alzheimer's disease and brain beta-amyloid deposition [271].

Reports of APOE ε polymorphism in PD susceptibility have been conflicting, mostly negative. Both ε4 and ε2 have been suggested to increase risk of PD [272-275] or associate with earlier age at onset [274, 276, 277]. Some studies have

indicated that ε4 might increase the risk of PD dementia or cortical Lewy body pathology [215, 278, 279].

4.2.3.4.4 Catechol-o-methyltransferase COMT

COMT is an intracellular enzyme present in soluble (s-COMT) and membrane-bound (MB-COMT) isoforms which function in elimination of biologically active or toxic catechols and some hydroxylated metabolites. COMT also acts as a detoxicating barrier between the blood and other tissues (e.g. in the intestinal mucosa and the brain) shielding against the detrimental effects of xenobiotics [108].

COMT gene is located on chromosome 22q. Methionine instead of valine at amino acid residue 158 (or 108 of S-COMT) results in thermolability and lower activity of the enzyme. Thus, this Val158Met polymorphism encoded by a G to A polymorphism in the corresponding codon in exon 4 of COMT gene determines the high and low activity forms of COMT [280]. Association of COMT Val158Met polymorphism has been extensively studied with various psychiatric conditions with inconclusive results [281]. Because of its role in dopamine metabolism and detoxication, COMT is a tempting candidate gene in PD. The role of COMT Val158Met polymorphism in the susceptibility to PD is controversial: both low [227, 229] and high [231, 282] activity alleles have been suggested to increase PD risk.

4.2.3.5 Genome-wide association (GWA) studies on sporadic PD

So far four GWA studies on sporadic PD have been published. In addition, two GWA meta-analyses have been made [283, 284]. An American study by Maraganore et al (2005) found 13 SNP's potentially important for determining the risk of PD [285], but subsequent case-control studies were not able to confirm any of these 13 associations [286, 287]. Foltynie et al reported association on chr 1p31 near PARK10 locus [288]. A GWA study in Dutch patients suggested association with chromosomes 9q and 14q and also suggested weak signal near MAPT and SNCA regions [289]. Fung et al studied in US patients 408 000 SNP's, none of which remained significant after correction for multiple testing

[290]. The meta-analysis by Evangelou (2007) did not find any significant associations either [283]. Fung and Maraganore have released their GWA data publicly available for future mining and augmentation.

Because of a large number (up to hundreds of thousands) of SNP's tested in a GWA study the risk of false positive findings is considerable. On the other hand, the commonly used Bonferroni correction for multiple testing may be too conservative and lead to false negative interpretations in GWA studies. GWA approach largely relies on the common disease - common variant hypothesis. Many important disease-causing or disease-predisposing variants may be rare and therefore not as readily detected by GWA approach. In addition, lack of information on gene function, insensitivity to structural variants, requirement for large samples, possible genotyping and case and controls selection biases, and high price are limitations of GWA studies [291]. Even though the associations found in GWA studies need to be confirmed by several other studies and large samples, GWA studies are effective means to find new candidate genes and generate new hypotheses.

4.3 Mitochondria and PD

The primary function of mitochondria is the production of cellular energy in the form of ATP by oxidative phosphorylation. They also have roles in controlling and mediating apoptosis, heme and steroid synthesis, free radical scavenging, calcium homeostasis and heat production [292]. Mitochondria are intracellular double-membrane organelles. The five complexes of the mitochondrial respiratory chain are located on the inner membrane. Mitochondria have their own maternally inherited genome. Mitochondrial DNA (mtDNA) is a circular double-stranded 16.5 kb DNA-molecule. However, majority of the mitochondrial proteins are encoded by nuclear DNA, synthesized in the cytosol and imported into mitochondria [293].

Classical experiments with MPTP (1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine) have suggested a role for respiratory chain defects in PD,

since MPTP exposure has resulted in dopaminergic neuron loss and parkinsonism through inhibition of mitochondrial respiratory chain complex I [294, 295]. Mitochondrial complex I deficiency in substantia nigra and platelets of PD patients has been reported [296, 297]. Many gene products of familial PD are implicated in mitochondrial function: PINK1 (PARK6), HTRA2/Omi (PARK13) and DJ-1 (PARK7) localize to mitochondria [298] and loss-of function mutations of *parkin* (PARK2) cause mitochondrial dysfunction and oxidative damage [299-301]. MtDNA deletions accumulate in the SN neurons along with aging, but mtDNA deletions are more abundant in the nigral neurons of PD patients than in age-matched controls [302, 303]. Certain specific mtDNA mutations or polymorphisms have been reported to associate with PD but this has not been consistently replicated [304-307]. Also specific mitochondrial haplotypes have been suggested to influence PD expression [308-310].

4.3.1 Mitochondrial DNA polymerase gamma (POLG)

POLG is responsible for the replication and repair of mtDNA. The catalytic α-subunit of POLG is encoded by a nuclear gene, *POLG1*, on chromosome 15q25. POLG consists of a N-terminal 5'-3' exonuclease domain, C-terminal polymerase domain and a 'spacer' region. Spacer region is involved in DNA-binding and in mediating the interaction with the accessory β-subunit, a processivity factor, encoded by a separate gene [311]. The consequence of *POLG1* defects is the accumulation of secondary mutations and deletions of mtDNA, which leads to progressive respiratory chain deficiency. In 2001 *POLG1* mutations were first connected to autosomal dominant progressive external ophtalmoplegia (PEO) [312]. Since then, a myriad of *POLG1* mutations with various clinical manifestations, e.g. Alper's syndrome (retardation, epilepsy and liver failure in infants), ataxia, neuropathy, myopathy, epilepsy, hepatopathy, valproate sensitivity, infertility, premature ovarian failure, and deafness, have been described [313-319]. Interestingly, also parkinsonism has been reported as a part of these *POLG* –associated syndromes [316, 317, 320-322]. The polyglutamine tract of *POLG1* is discussed later.

4.4 Interaction of genes and environment

Complex diseases such as PD are widely believed to result from interplay of genetic predisposition and environmental risk factors. The individual genetic effects may be small and difficult to detect consistently [323]. It has been proposed that inconsistency in genetic associations might be due to unrecognized interactions with environmental factors or other genes [324]. Therefore focusing on gene-environment or gene-gene interactions is of growing interest.

Coffee consumption and smoking have consistently been found to be protective against PD. In a large meta-analysis a pooled relative risk of 0.59 was calculated for ever-smokers vs. never-smokers, and 0.69 for coffee drinkers vs non-coffee drinkers [135]. Recently McCullough et al (2008) studied the interactions of smoking and drinking coffee with genetic factors such as APOE ε2/3/4, MAPT H1/H2, and SNCA promoter repeat Rep1 alleles. In their material APOE ε2 was associated with nearly two-fold PD risk in those who did not drink coffee, but this predisposing effect of ε2 waned with increasing coffee consumption. They also suggested interaction between SNCA Rep1 allele length and smoking [324]. Clearly these findings should be considered hypotheses that demand verification, but they serve as examples of the possible gene-environment interactions. There is previous evidence for a putative interaction of APOE with smoking and alcohol consumption in the risk of cognitive decline [325]. There are, however, no precedents of the interaction between SNCA and smoking.

A gene-gene synergistic interaction in PD risk has been suggested between the *MAPT* (*tau*) H1 haplotype and *SNCA* (SNP rs356219 from the 3' region of *SNCA*). By studying 650 PD patients from the UK, Goris et al (2007) found that carrying a risk genotype at either of these loci modestly increased the risk for development of PD. Odds ratios (OR) were 1.34 ($p=0.002$) and 1.45 ($p=0.003$) for the *MAPT* H1/H1 and *SNCA* rs356219 G/G genotypes. The

combination of risk genotypes at both loci approximately doubled the risk (OR 2.14, p=0.000003) [221]. There is biochemical evidence for an interaction of the protein products tau and α-synuclein. It has been shown they can seed each other's aggregation at low protein concentrations [326]. There is increasing clinical and neuropathological evidence suggesting that synucleinopathies and tauopathies are linked [327, 328].

Possible interaction of herbicides and genes encoding detoxicating enzymes has been proposed. Glutathione transferases (GST) are a family of inducible phase II enzymes that metabolize xenobiotics, including pesticides. GSTP1 haplotypes were reported to associate with an increased PD risk in subjects exposed to pesticides [252]. Another study suggested that herbicide exposure modifies the effect of GSTP1 haplotypes on the age of PD onset [249]. Also GSTM1 null subjects heavily exposed to solvents may be at increased PD risk [251]. The debrisoquine hydroxylase (CYP2D6) metabolizes several xenobiotics, including organophosphate pesticides and MPTP. CYP2D6 activity is genetically determined. Being a "poor metabolizer" is a recessive trait. 5-10% of white population is poor metabolizers with undetectable CYP2D6 activity. In a French study, among subjects exposed to pesticides, the risk of PD was increased twofold among CYP2D6 poor metabolizers in comparison with non-poor metabolizers. Pesticides had a modest predisposing effect in subjects who are not poor metabolizers. On the other hand, poor metabolizers were not in increased PD risk in the absence of pesticide exposure [242]. These examples fit into the hypothesis where a genetic predisposition (genetic variation/defect in a detoxicating enzyme) exists resulting in toxic injury to the neurons and the development of PD.

4.5 Hypotheses on the pathogenesis of PD

Although the pathogenesis of sporadic PD is still a mystery, discovery of the genes of familial PD have highlighted some pathways involved in nigral degeneration, including altered or increased α-synuclein (SNCA), impaired

protein degradation and protein aggregation, mitochondrial dysfunction, and oxidative stress. Also excitotoxicity and inflammation may contribute to the pathogenesis [329].

SN has a high propensity for oxidative stress and is deficient in protective mechanisms [330]. The susceptibility of dopamine (DA) and its metabolites to produce reactive oxygen species (ROS) might explain the vulnerability of the SN neurons [331]. In addition, SN is rich in iron and copper, and the oxidation-reduction cycle of iron can produce free radicals. Mitochondrial dysfunction, which leads to uncoupling of redox reactions and production of ROS, has also been implicated in PD [298] and is discussed above in its own chapter. Some evidence from post-mortem studies suggests that the SN might be short of antioxidant molecules, e.g. glutathione [332]. Of the familial PD loci, PINK-1 and DJ-1 seem to be involved in the responses to oxidative stress, and their mutations might therefore lead to defective oxidative stress tolerance [333].

Another explanation for SN involvement could be the dopamine transporters (DAT) on dopaminergic cells [334]. MPTP and 6-hydroxydopamine that produce experimental parkinsonism enter the dopaminergic neurons via DATs, and in transgenic mice even modest differences in the expression of DAT influence the dopaminergic neuronal damage caused by experimental toxins [335, 336]. Mice that express no DAT are resistant to MPTP [337]. DAT might also be the route of environmental toxins to the SN.

Considerable evidence, such as mutations or multiplications of *SNCA* in familial PD and allelic association with sporadic PD, and the role as major component of LB's and Lewy neurites, suggest involvement of SNCA in the pathogenesis of PD. This is supported by the results of Dauer et al: SNCA null mice were shown to be resistant to neurotoxicity of MPTP [338]. Increased localization of SNCA to the nucleus, by mutations in or increased expression of SNCA, might be toxic to neurons through decreased histone acetylation, possibly leading to aberrations in transcriptional control, and ultimately to cell death [339]. Phosphorylation of SNCA may affect its neurotoxicity, possibly by causing

increased nuclear localization of SNCA [340]. Mutations in or over-expression of SNCA may lead to abnormal fibril formation and aggregation of SNCA [152, 341]. Adult-onset loss of dopaminergic neurons, intraneuronal SNCA-containing inclusions, and a movement disorder were seen in a *Drosophila* model of mutant SNCA [342]. SNCA aggregation may also be promoted by free radicals, and dopamine metabolites [343, 344]. It has been suggested that actually smaller oligomers of SNCA, protofibrils or Lewy neurites may be the culprits in neuronal degeneration whereas LB's might be relatively inert dumping grounds [344], analogous to Alzheimer's disease [345].

LRRK2 and PINK1 both encode protein kinases, and might be involved in phosphorylation of proteins that accumulate. Pathogenic mutations seem to increase the kinase activity of LRRK2 *in vitro*, which may suggest a toxic gain-of-function mechanism [346, 347]. Their substrates are not known, but their indirect targets have been suggested to include SNCA and tau, phosphorylated species of which have been associated with toxicity and accumulation as LB's or Lewy neurites and neurofibrillary tangles, respectively [348].

Proteolytic stress may underlie cell death in PD. Normally abnormal, damaged, misfolded or otherwise unwanted proteins are degraded by the ubiquitin-proteasome (UPS) system [349]. Tagging these proteins with ubiquitin signals their transport to the proteasome for degradation. If production of misfolded protein exceeds the degradation capacity of the UPS, proteolytic stress arises and may lead to accumulation of these proteins and impairment of cellular functions. Of the gene products of familial PD, SNCA, UCHL1 and parkin can be theoretically linked to proteolytic stress. Parkin (a ubiquitin E3 ligase) and UCHL1 (ubiquitin carboxyterminal hydrolase) operate in the UPS. Mutations in them may lead to impairment in UPS and accumulation of proteins, including SNCA [329]. The ability of parkin to ubiquitinate protein might also be impaired by S-nitrosylation resulting from exitotoxic damage [350]. Proteasomal impairment has been observed in post-mortem studies on sporadic PD [351]. It is possible that proteasomal dysfunction is not a primary phenomen in PD but secondary to oxidative stress or mitochondrial dysfunction [329].

Autophagy-lysosome pathway (ALP) is another means to degrade cytosolic and misfolded proteins, and impairment of ALP may also lead to accumulation and aggregation of proteins, resulting in cellular toxicity. ALP is considered the default pathway when an aggregate-prone substrate cannot be degraded by the proteasome. Three forms of ALP are macroautophagy, chaperone-mediated autophagy (CMA) and microautophagy [352]. Wild-type SNCA can be degraded both by the UPS and by ALP, but mutated SNCA cannot be degraded by the CMA : although it binds to the lysosomal membrane with high affinity, it does not translocate into the lysosome. Mutant SNCA, by its high-affinity binding, also seems to block uptake and degradation of other substrates by the lysosome, thus causing a more general CMA blockage [352]. SNCA mutations are rare but more common are posttranslational modifications of SNCA, by e.g. products of dopamine metabolism, which may impair degradation of SNCA by CMA [353]. Mutations in ATP13A2 (PARK9), a lysosomal ATPase, lead to a failure of autophagy and aggregation of SNCA, further supporting the role of ALP dysfunction in neurodegeneration [354].

Even though there is evidence supporting all these theories, the pathogenesis and preferential neuronal vulnerability in PD remain unknown. Hypotheses on PD pathogenesis are summarized in Figure 3.

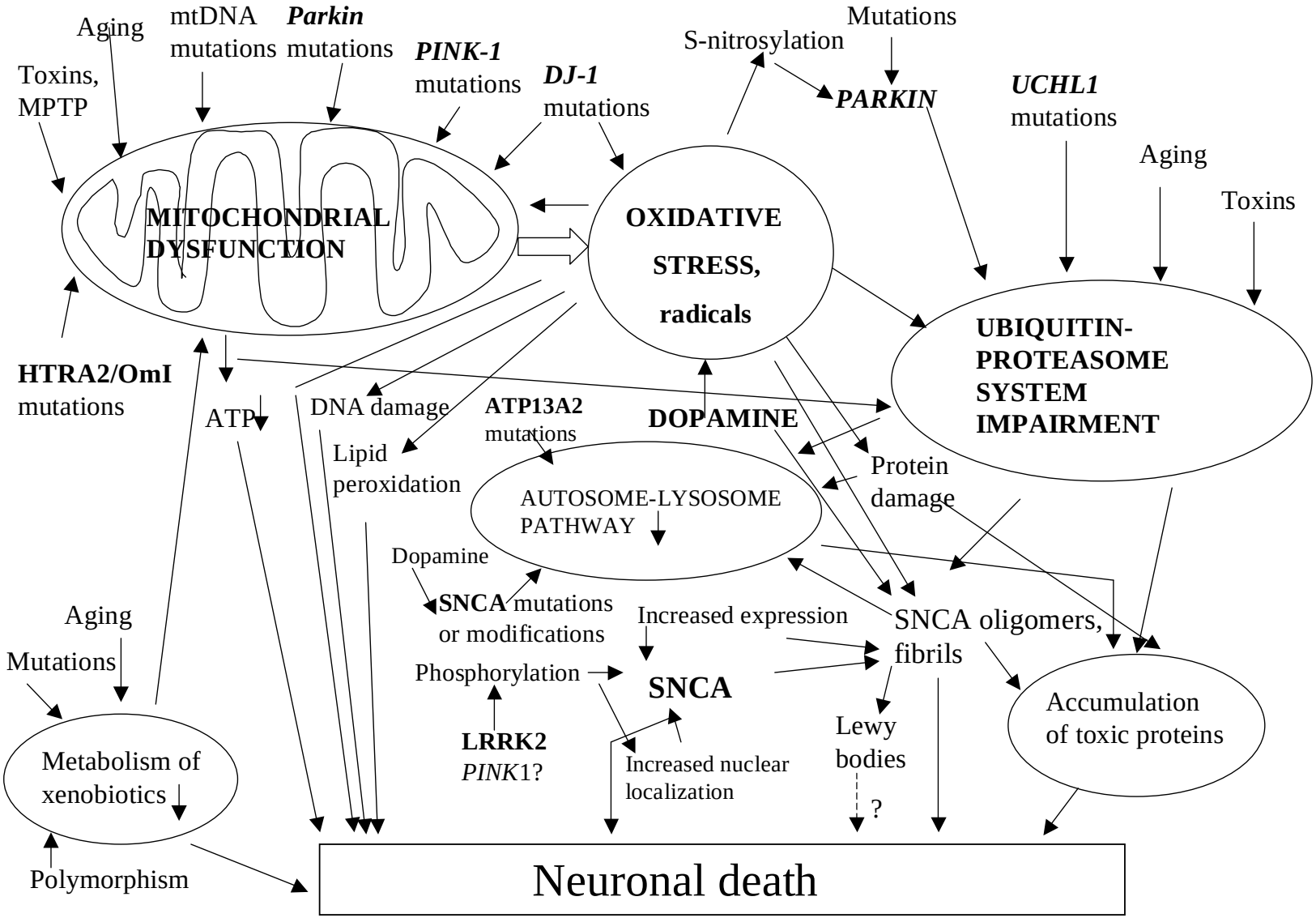

Figure 3. Hypotheses on the pathways and their connections in PD pathogenesis

AIMS OF THE STUDY

The pathogenesis of PD is still largely unknown, and PD lacks neuroprotective therapy. Knowledge on the pathogenesis would help development of neuroprotective and even preventive treatments. Finding susceptibility genes of PD helps to elucidate the pathogenesis.

The specific aims of this study were:

- To assess the accuracy and usefulness of ^{123}I-β-CIT SPECT in PD diagnosis in routine clinical practice (Study I)

- To study the association of PD and APOE ε2/3/4 polymorphism, previously reported to affect susceptibility to or prognosis of various neurological conditions, as well as to study the association of PD and APOE promoter and enhancer polymorphisms, and APOE ε3 haplotypes, the last three not previously studied in PD (Study II)

- To study the association of *COMT*, involved in dopamine metabolism, and PD (study II)

- To test association of *parkin* and *DJ-1,* loci of familial PD, and sporadic PD (studies II, III)

- To study the role of *POLG1* variants in patients with PD, as mitochondrial dysfunction has been suggested in the pathogenesis of PD and parkinsonism was reported in PEO families with *POLG1* mutations (studies IV and V)

MATERIALS AND METHODS

1. THE ^{123}I-β-CIT SPECT STUDY

1.1 ^{123}I-β-CIT SPECT patients

In the study I, we included all 185 consecutive patients of the outpatient clinic of the Department of Neurology, Helsinki University Central Hospital, who underwent ^{123}I-β-CIT SPECT based on clinical history and/or neurological examination from 1996 to spring 1999. All had also MRI and/or CT. The most likely clinical diagnosis was made based on standard clinical criteria, clinical follow-up of at least 2-4 years after SPECT imaging, and MRI and/or CT findings. Hoehn-Yahr and Schwab-England scales were used to assess the disease stage of PD patients.

Of the 185 subjects (83 women/102 men) scanned with ^{123}I-β-CIT SPECT, 92 patients had PD and 16 had essential tremor (ET). 15 patients with vascular parkinsonism (VP) had either a focal vascular lesion in the basal ganglia area, general leukoaraiosis or both, resistance to antiparkinsonian medication, and a clinical picture typical to VP. 8 patients with MSA and 4 with PSP were together grouped as a P+ group (N=12) in the statistical analysis. Of the 12 patients with drug-induced parkinsonism, neuroleptics were regarded causative in eleven and lithium in one. 5 patients had probable DLB, and 5 cervical or other type of focal dystonia. 20 patients had fears or complaints suggestive of an extrapyramidal disease, but had no unequivocal signs on neurological examination and MRI was normal. In addition, clinical follow-up did not confirm striatal dysfunction and the symptoms resolved during follow-up. Eleven of them were diagnosed as having psychogenic parkinsonism. Nine similar patients were not evaluated by a psychiatrist and therefore had no official diagnosis of somatoform disorder, but we consider all twenty having psychogenic parkinsonism. However, the numerical data of these subjects were used only descriptively and not used for statistical analyses as they are not an optimal control group. 8 patients (Alzheimer's disease, cervical disc prolapse, neurosyphilis, normal-pressure hydrocephalus, sequlae of radiculitis, post-

traumatic extrapyramidal symptoms, and 2 cases with ataxia NUD) did not fit into any of the groups above and were therefore excluded from the statistical analysis. Thus, the total N in the analyses is 177.

1.2 ^{123}I-β-CIT SPECT methods

SPECT scanning was performed 24 hours after an intravenous injection (185 MBq on average) of ^{123}I-β-CIT. Before the tracer injection, thyroidal uptake was blocked by 400 mg of oral potassiumperchlorate. A Picker Prism 3000XP triple-head gamma camera with a low-energy, ultrahigh resolution (LEHR) fan beam collimator (resolution 6.7 mm at 10cm distance) was used. Data were reconstructed using a ramp filter and 3D postfiltering with a low pass filter. Chang attenuation correction was performed. 4.4 mm thick transaxial slices were reformatted to the orbitomeatal line. Regions of interest (ROI) were drawn on the striatum and separately on the caudate and putamen by using a pre-defined ROI model, the shape and rotation of which was adjusted for each measurement, and counts-per-pixel values were calculated for each ROI. A ROI drawn on the cerebellum was the reference region with unspecific binding. The striatal ^{123}I-β-CIT uptake was expressed as the mean striatal counts divided by cerebellar mean counts.

1.3 Statistical methods

The parametric analysis of variance (ANOVA/MANOVA) was used to analyse the statistical significance of difference in means between the various diagnostic groups. A p-value < 0.05 was considered significant. Tukey's honest significant test for unequal N was used to compare the mean uptake values in the patient groups. Non-parametric Spearman rank correlation test was used to calculate correlations.

The cut-off points for analyses of sensitivity and specificity were calculated using a ROC-analysis with emphasis on high sensitivity. The sensitivity and specificity of ^{123}I-β-CIT was calculated in a 2 x 2 contingency table where sensitivity was defined as the proportion of test positive cases out of all PD

cases, and specificity as the proportion of test negative cases out of all PD-negative cases.

2. GENETIC ANALYSES

2.1 Patient inclusion criteria

We collected a case-control series (II-IV) of idiopathic apparently sporadic PD patients (N=147, 87 men/60 women) who had clinically typical PD from the outpatient clinics of the Department of Neurology, Helsinki University Central Hospital (96 patients) and Seinäjoki Central Hospital (51 patients). The PD diagnoses were verified according to UK Brain Bank Criteria with a clinical follow-up for at least 4 years or, alternatively, clinical follow-up for at least 2 years plus ^{123}I-β-CIT-SPECT findings supporting idiopathic PD. Demented and atypical patients, as well as patients who reported first degree relatives with parkinsonism were excluded. Mean age of the patients was 67.2 years (range 38-88 years) at the time of sample collection.The patients' neurologically healthy spouses (N=137, 50 men/87 women) were recruited as controls.

In study IV, also two other control groups were used for the POLG CAG /polyQ repeat analysis. Population controls (n=516) consisted of the previously published Vantaa 85+ subjects (n=515, successful POLG poly-Q sequence obtained in 447) [355] and healthy blood donors (N=69).

In study V, we used previously published publicly available PD and neurologically normal Caucasian control samples from NINDS repository (patient plates NDPT001, NDPT005, NDPT007, NDPT014, NDPT015, NDPT016, NDPT017, NDPT018 and control plates NDPT002, NDPT022, NDPT023, and NDPT024) (http://ccr.coriell.org/Sections/Collections/NINDS/DNAPanels.aspx?PgId=19#) . Of them, we excluded 24 patients who had other likely pathogenic mutation (10 with parkin, 10 with LRRK2, and 4 with PINK1 mutation). A PSP patient, erroneously in this sample collection, was excluded from our study. Sequencing was successful in 652 patients (44 % females/ 56 % males), all of

them Caucasians. The mean age of the PD patients was 65.3 (range 29-88) years, and the mean age-at-onset (AAO) 56.9 (range 7-87) years.
Sequencing was successful in 292 control samples (51% females/49 % males). The mean age of the controls was 68.2 (range 55-95) years.
Additional controls retrieved from two other sources, where *POLG1* polyQ (CAG-repeat) alleles had been defined by sequencing, were also used in comparisons: mostly Caucasian controls (n= 605) from the doctoral thesis of Anja Rovio (2006) http://acta.uta.fi/english/teos.phtml?10863 , p.60 , and pooled Finnish controls from IV (n= 644, spouses and population controls).

2.2 DNA methods

DNA was extracted from blood leukocytes using standard phenol-chloroform method.

II The genetic markers were amplified by PCR, digested with an appropriate restriction enzyme and electrophoretically separated on agarose gels. Marker alleles were visualized with ethidiumbromide by UV light.
For typing APOE ε2/3/4 polymorphism, previously published primers and digestion enzyme HhaI were used [356]. Additional SNP's from APOE were also genotyped: -219G/T (Th1) polymorphism in APOE promoter area (digestion with BstNI), and +113G/C (IE1) in intron 1 (NlaIV).
PARKIN SNP genotypes Ser167Asn (AlwNI, exon 4), Arg366Trp (NciI, exon 10) and Val380Leu (Bsp1286I, exon 10) were determined as previously described [203].
COMT Val158Met polymorphism was genotyped using previously published primers and Nla III digestion [357]. COMT SNP rs6270 was digested with BsmFI. The primers used for amplification of rs6267 were 5'-AGCATGCGGAGCCCGGGAAT*-3' and 5'-CTGGAAATGGGGTGATAACAGC-3'.
A restriction site for BsmI was generated by a mutation (C->T) on the last base (*) of the forward primer. Digestion of the G-allele yielded 170bp and 23bp fragments whereas T-allele had no restriction site for BsmI.

III PCR for *DJ-1* exon 1 g. 168_185del was performed using primers

5'-GAGGTAGACTCGGCCGGAC-3' and 5'-TTCTGGACGCTTCAGCGTTG-3' The 405 bp ins allele product and the 387 bp del allele were visualized with ethidium bromide and UV on a 3% agarose gel.

IV, V The sequencing of the *POLG1* exons was performed using previously published intronic primers [312]. The fragments were sequenced by automated nucleotide sequencing with the BigDye terminator Ready Action Kit v 1.1 and a 3100 Genetic Analyzer automatic sequencer. The primers for the SNP's rs2238297 C/T and rs2302084 A/G were designed and restriction enzymes searched using SciEd software. The PCR products were digested with the restriction enzymes Tsp45I and MspI, respectively.

2.3 Statistical methods

In the genetic studies, χ^2 or Fischer exact test were used to compare allele or genotype frequencies between cases and controls. $P < 0.05$ was considered significant.

Haplotype frequency estimation was carried out with the expectation-maximization algorithm by using HAPLO [358] and SNPHAP software http://www.gene.cimr.cam.ac.uk/clayton/software/snphap.txt . HAPLO was used to estimate haplotype frequencies in PD and control groups and to calculate global p-values for association with PD whereas SNPHAP was used to analyze the association of individual haplotypes with PD.

3. ETHICS

The study has been approved by the Ethics Committee of the Department of Neurology, Helsinki University Central Hospital (65/1999) and by the Ethics Committee for Ophthalmology, Otorhinolaryngology, Neurology and Neurosurgery in the Hospital District of Helsinki and Uusimaa (439/E9/2000, 90/2001). The patients and controls gave their written informed consent.

RESULTS AND DISCUSSION

1. ^{123}I-β-CIT SPECT IN THE DIFFERENTIAL DIAGNOSIS OF PD (I)

Many studies had assessed ^{123}I-β-CIT SPECT in PD patients vs. controls, or other predefined case-control-setting with good results. This method's ability to differentiate between numerous diagnostic possibilities in clinical practise had not been assessed in a single study. We assessed the accuracy and clinical usefulness of ^{123}I-β-CIT SPECT in the differential diagnosis of 177 consecutive patients with signs or symptoms suggesting possible extrapyramidal disease.

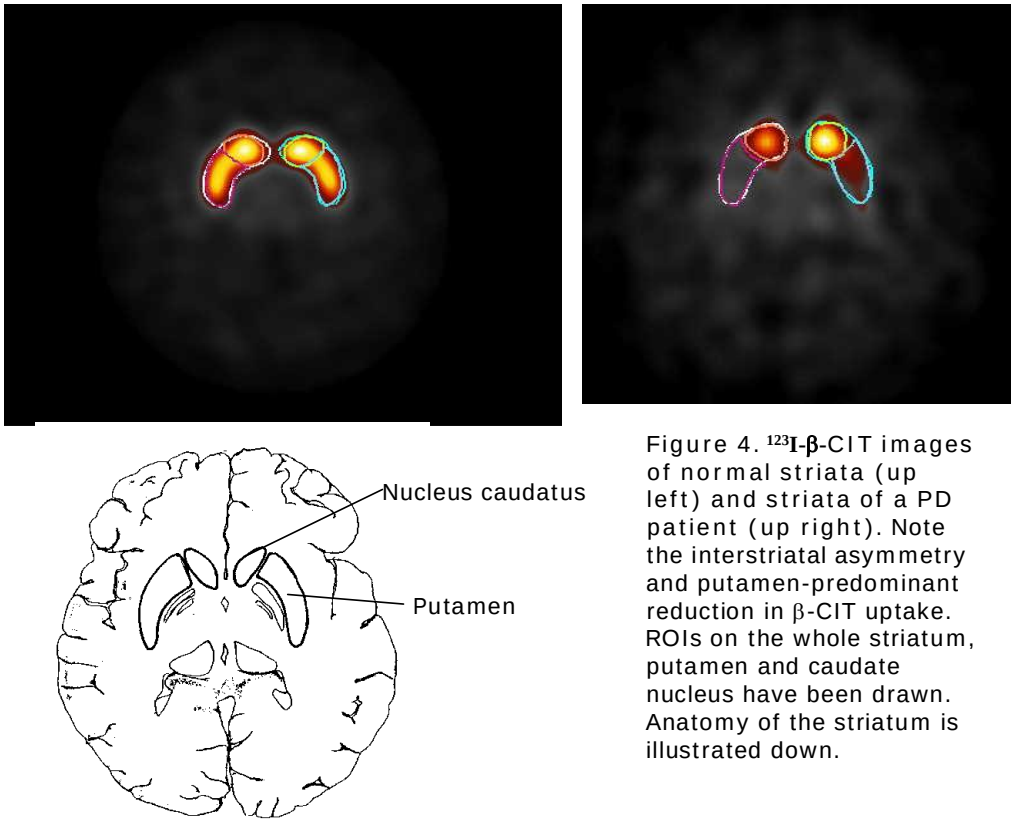

Figure 4. ^{123}I-β-CIT images of normal striata (up left) and striata of a PD patient (up right). Note the interstriatal asymmetry and putamen-predominant reduction in β-CIT uptake. ROIs on the whole striatum, putamen and caudate nucleus have been drawn. Anatomy of the striatum is illustrated down.

1.1 ^{123}I-β-CIT uptake in different movement disorders

The striatal β-CIT uptake in all the patient groups is visualized in figure 5. The means in all ROIS in all groups are presented in table 2 of study I and were

not copied here. β-CIT uptake was significantly lower in PD patients than in patients with DIP, essential tremor or dystonia both in the whole striatum or in the putamen and in the caudate nucleus. The uptake to the whole striatum or to the caudate and putamen was similar in the patients with DIP, dystonia, essential tremor, and psychogenic parkinsonism.

In the P+ and DLB groups the DAT density was low and the striatal β-CIT uptake ratios overlapped with those of the PD group. No significant difference was found between these groups. Some studies have suggested there may be difference in the caudate:putamen ratio between PD and P+ or DLB groups, since in PD the degeneration of the projections to the putamen predominates and projections to nucleus caudatus are relatively spared compared to PSP or MSA, in which the striatum may be more uniformly affected. Many have failed to find a difference [78, 79, 81, 359]. Among our patients the caudate:putamen ratio was highest in the PD group but it was not significantly different from P+ or DLB groups (data not shown). Also interstriatal asymmetry seemed to be more pronounced in PD patients but did not allow differentiation of these disorders, in line with most previous studies [78, 79, 82, 84].
Thus, by using ^{123}I-β-CIT SPECT only, PD, PSP, MSA and DLB can be differentiated from essential tremor and DIP, but not reliably from each other. Recently some studies have proposed that quantification of midbrain DAT signal, in addition to striatal, might add the value of β-CIT SPECT in the differential diagnostics between PD and P+ [360, 361].

The striatal uptake of β-CIT in the vascular parkinsonism (VP) group was variable (range 3.6 – 6.3) and the mean DAT density was between that found in psychogenic parkinsonism, essential tremor, dystonia, drug-induced parkinsonism and in the PD group. In VP the mean β-CIT uptake ratios in the whole striatum, caudate and putamen were significantly higher than in PD patients but lower than in the patients with psychogenic parkinsonism. (Fig 5) In half of the cases β–CIT uptake was within normal range and below the normal range in the rest. Gerschlager et al have suggested VP may be differentiated from PD using β-CIT SPECT [92]. However, closer inspection of

their data also shows overlap between normal and pathological range. Consequent results of FP-CIT studies on DAT density in VP patients have been conflicting [93, 94].

Remarkable interstriatal asymmetry of DAT density, more typical to PD, could also be seen in some of our patients with VP. A vascular lesion was seen in the corresponding basal ganglia region in the MRI or CT scans of those asymmetrical cases. In patients with degeneration of deep white matter without focal basal ganglia lesions uptake was essentially normal. Thus, differentiating an extrapyramidal syndrome caused by a focal striatal vascular lesion from PD by means of ^{123}I-β-CIT SPECT is not reliable without CT or MRI. As white matter degeneration in the absence of striatal lesions does not seem to result in a significant decrease in DAT density, ^{123}I-β-CIT SPECT seems to differentiate PD from VP in such cases.

It is of note that DAT imaging in drug-induced parkinsonism (DIP) has been reported previously in only a small number of cases. Mostly normal FP-CIT uptake indices in small groups of DIP patients have been reported. β-CIT SPECT in DIP has not been reported at all. We confirmed the previous FP-CIT results of normal indices with β-CIT SPECT in this patient group. In some patients with irreversible DIP, diminished DAT density has been found, suggesting possible exacerbation of underlying neurodegenerative parkinsonism in these patients [95-97]. Such cases were not found among our patients.

In our patient material, the role of ^{123}I-β-CIT SPECT was important in the diagnosis of some patients with DIP, some cases with psychogenic symptoms, and some patients with atypical essential tremor.

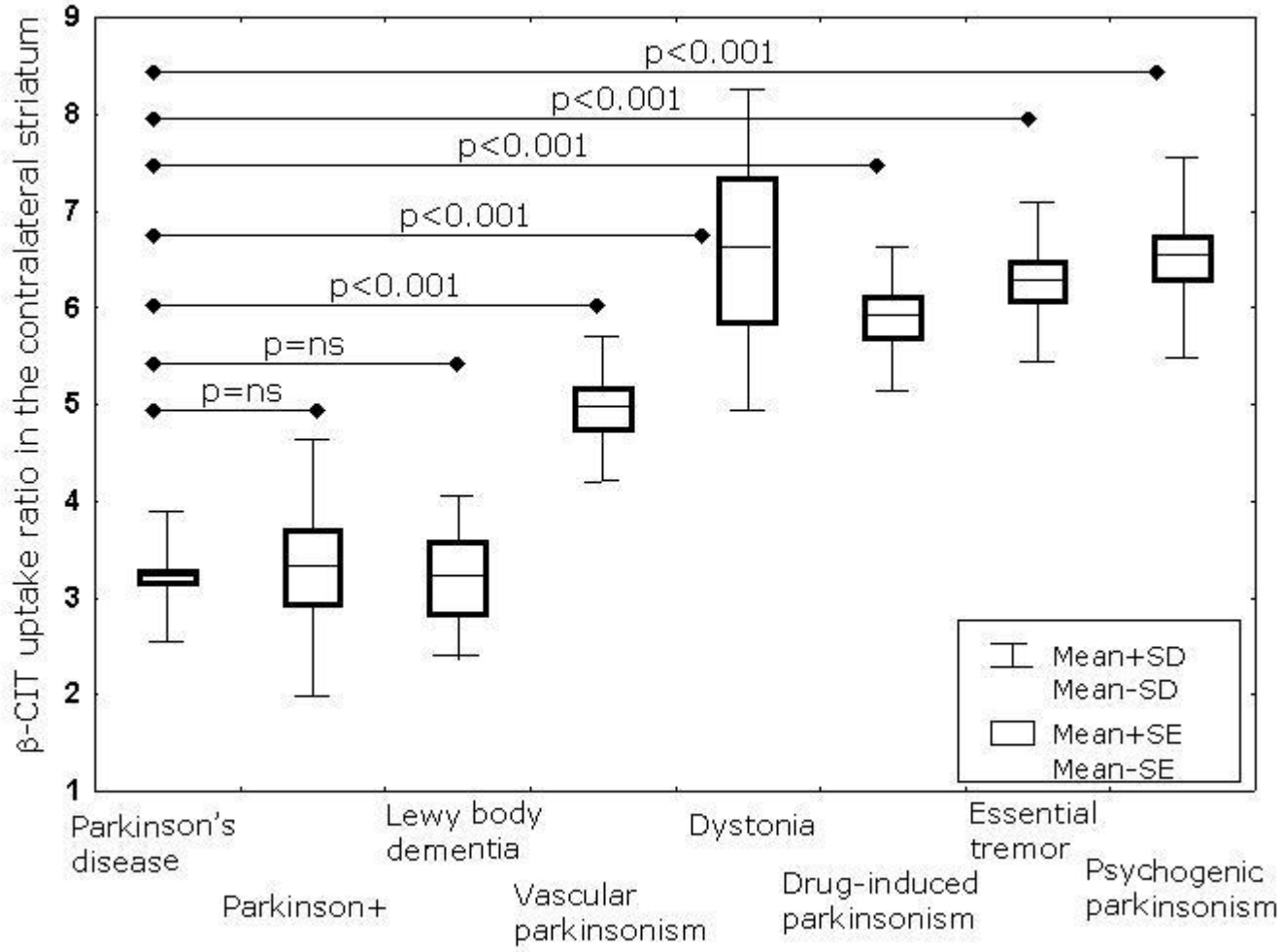

Figure 5. Striatal ^{123}I-β-CIT uptake in different patient groups

1.2 Sensitivity and specificity for PD are influenced by age

Sensitivity and specificity were analysed to address the question how ^{123}I-β-CIT SPECT differentiates idiopathic PD form other conditions. The sensitivity and specificity in PD diagnosis in both the contralateral striatum (as whole) and the contralateral putamen were 98.9 % and 80.5 %, respectively. The uptake ratios in the contralateral striatum and putamen gave better sensitivity and specificity than those in the caudate nucleus or ROIs on the ipsilateral side. (Table 5)

Table 5. Sensitivity and specificity of ^{123}I-β-CIT SPECT in the diagnosis of PD

	ALL		<55 YEARS		≥55 YEARS	
	Sensitivity *(%)*	Specificity (%)	Sensitivity (%)	Specificity (%)	Sensitivity (%)	Specificity (%)
CONTRALATERAL STRIATUM	98,9	80,5	100	100	98,4	68,5
IPSILATERAL STRIATUM	93,5	79,3	100	92,9	92,2	70,4
CONTRALATERAL CAUDATUS	93,5	74,7	100	96,4	90,6	63,0
IPSILATERAL CAUDATUS	92,4	74,7	100	89,3	93,8	59,3
CONTRALATERAL PUTAMEN	98,9	80,5	100	100	100	68,5
IPSILATERAL PUTAMEN	96,7	81,6	100	96,4	96,9	72,2

The specificity was remarkably higher in subjects younger than 55 years than in the subjects older than 55 years. In patients <55 years, both sensitivity and specificity were 100 % when the contralateral striatal/cerebellar uptake ratio cut-off was set at 4.5 and also 100% in the contralateral putamen with the putaminal/cerebellar cut-off value of 3.1.

In patients >55 years the sensitivity of the contralateral striatum was 98.4% and specificity 68.5% using the same cut-off value of 4.5. Uptake measured from the contralateral putaminal ROI yielded 100% sensitivity and 68.5% specificity (cut-off 3.9). The cut-off age limit of 55 years was arbitrary and best divided the material into good and poorer specificity.

The age-dependent difference in the diagnostic accuracy of β-CIT is likely explained by different distribution of diagnoses in the old and in the young. The age-related decline in β–CIT uptake was in this material 4 % per decade. This decline, however, is not high enough to affect specificity. As the diagnoses that diminish specificity and are present in the older population – P+, VP and DLB – have β-CIT uptake ratios similar to those of PD, using age-corrected values would not increase specificity. In younger subjects VP and DLB are rare. It is likely that in a larger sample of younger patients cases of abnormal β-CIT uptake with other diseases than idiopathic PD would exist. However, our result suggests that in real life the accuracy of β-CIT in this group is expected to be close to 100%. A patient younger than 55 with extrapyramidal symptoms is very likely to have PD if reduced DAT density is found in the striatum. However, the majority of patients with suspected nigrostriatal pathology are older than 55.

To be able to exclude or show presynaptic nigrostriatal degeneration (including PD, P+ and DLB) as such and differentiate these conditions from conditions without nigrostriatal presynaptic pathology, e.g. DIP, essential tremor, psychogenic parkinsonism, Alzheimer's disease and normality, is also of value. In differentiating conditions with known presynaptic pathology from those without, the specificity of β-CIT SPECT would be better than in PD diagnosis, but the presence of VP especially in older age would diminish the specificity also in this setting.

1.3 Correlation of β-CIT with age and severity of symptoms

β-CIT uptake correlated negatively with age ($r=-0.39$, $p<0,01$) in non-parkinsonian subjects (psychogenic parkinsonism, essential tremor, dystonia and drug-induced parkinsonism). The age-related decline in β-CIT uptake was around 4 % per decade in this material, which is concordant with previous reports [53, 71, 362]. In PD, the nigrostriatal degeneration caused by the disease process is undoubtedly more prominent that age-associated nigrostriatal

degeneration. Hence, the correlation between age and reduction in DAT density was not significant in the PD group ($r=-0.18$ $p=0.08$).

In PD patients the decrease in striatal β-CIT binding correlated significantly ($p<0.01$) with symptom severity. The correlation between the patients' Hoehn and Yahr stage and β-CIT uptake was significant in the whole striatum as well as in the putamen and caudate ROIs on both the contralateral and ipsilateral side. Striatal β-CIT uptake correlated also with the patients' Schwab & England scores in all ROIs. The correlations with Hoehn-Yahr and Schwab-England were constantly mildly stronger in the ipsilateral side than in the contralateral side. Thus, the better-preserved striatum seemed to determine the patient's functional capacity rather than the more severely affected side. (Data not shown.)

In patients with unilateral parkinsonism (Hoehn-Yahr stage I) the reduction in β-CIT uptake was more prominent in the contralateral side, but the average ipsilateral DAT density was also clearly decreased (–37% compared to the group with psychogenic parkinsonism). Thus, ^{123}I-β-CIT SPECT is able to detect PD in preclinical phase and may, therefore, be used in clinical trials to detect PD very early or even presymptomatically, as a more available and cheaper alternative for PET. Especially in the future, when neuroprotective agents hopefully will be available for clinical use, this may be important for at-risk individuals. Here we also used ^{123}I-β-CIT SPECT as a part of PD patient selection for the genetic studies.

2. APOE, COMT and parkin genes in sporadic PD (II)

2.1 *APOE* in PD

APOE ε2/3/4 polymorphism in PD risk

In our Finnish case-control dataset we did not find any association between APOE ε2/3/4 polymorphism and PD. Both allele and genotype frequencies were similar in cases and controls (Table 6).

Previously both ε4 and ε2 have been suggested to increase the risk of PD in some studies [214, 272-274, 363, 364] while many groups including ours have found no association [216-220, 365-367]. At present it is not clear, whether the initial positive findings have been true or false positives. If they have been true, the discordance between studies may be due to genetic heterogeneity between the populations studied. As PD constitutes a spectrum of symptoms, the clinical features tend to vary from study to study.

The development of dementia, Alzheimer-type neuropathology or the amount of cortical LB's in PD patients have also been proposed to associate with the ε4 allele [215, 279, 368-370]. None of the PD patients included in our study were demented at the time of recruitment. It is possible that the contribution of APOE, or a gene in its vicinity, is different in striatal and cortical pathologies.

APOE ε2/3/4 polymorphism and age-of-onset of PD

ε4 allele has also been suggested to lower age at onset (AAO) of PD [274, 276, 277]. The AAO of all our patients was not precisely known. However, we had 11 early-onset PD patients in our material who definitely had AAO ≤ 45 years. Among them, 4 (36%) had ε2/ε3, 4 (36%) ε3/ε3, and 3(27 %) ε3/ε4 genotype. Frequency of ε3/ε4 genotype was similar to that in the rest of our patients and in the controls (see table 6). Thus, ε4 was not overrepresented in young-onset cases. Frequency of the ε2/ε3 genotype was somewhat higher than expected in our early-onset patients (36% vs 10 % in controls). Maraganore et al [218] had a similar finding, but our group of early-onset patients is too small to draw any conclusion.

APOE promoter and enhancer polymorphisms (unpublished data)

APOE promoter (Th1/E47cs, -219 G/T) and enhancer (APOE intron 1 enhancer element, IE1, +113 G/C) polymorphisms have also been reported to modify the risk of Alzheimer' disease [271, 371-374]. However, it has remained unclear whether the effects of these polymorphisms are independent, by modifying the level of APOE expression, or reflect LD with APOE ε2 and ε4 alleles. Myllykangas et al [271] studied APOE haplotypes using these above-mentioned polymorphisms in ε3 homozygous subjects to control the effect of LD with the predisposing ε4 and protective ε2 alleles and found that the effects of these polymorphisms were seen independently of the e2 and e4 alleles. In their study on neuropathologically verified Alzheimer's disease, the frequencies of the –219T-ε3 and +113C-ε3 haplotypes were significantly lower, and -219G- ε3 and +113G- ε3 higher in patients compared to controls.

Association between –219 G/T polymorphism and PD has been studied in only one study [375] and neither +113G/C polymorphisms nor APOE haplotypes have been studied in PD patients. We genotyped these polymorphisms and constructed APOE haplotypes (unpublished data), and found allele and genotype frequencies of these markers very similar in our PD and control groups (table 6). Neither were APOE haplotypes associated with PD. The two-point haplotype data on ε3 homozygous subjects are presented in table 7. Also 3-marker haplotypes with markers –219G/T – +113G/C – APOE were analysed with HAPLO software both in the whole material and also in ε3 homozygotes. Haplotype distributions were similar in cases and controls (HAPLO p=1.000, data not shown).

The risk of Alzheimer's disease is strongly associated with APOE alleles, with ε4 increasing and ε2 decreasing susceptibility [164], and the effect of different ε3 haplotypes has been shown [271]. Since Alzheimer's disease and PD are both neurodegenerative diseases and they share some neuropathological features [24-26, 376], common neuropathological mechanisms for these disorders have been

investigated. Based on previous and the results presented here, neither APOE ε polymorphism nor the APOE region haplotypes are a common denominator. Although APOE region does not seem to have a major role in PD susceptibility, its role in clinical subgroups and as an age-of-onset modifier deserves further study.

Table 6. APOE, -219G/T and +113G/C polymorphisms in PD patients and controls

APOE				
	PD patients		Controls	
	n	f	n	f
ε2	21	7 %	22	8 %
ε 3	222	76 %	196	72 %
ε 4	51	17 %	56	20 %
Σ	294		274	
	χ2=1,17 2 df p=0,56			
ε2 / ε2	1	1 %	1	1 %
ε 2 / ε3	16	11 %	14	10 %
ε 2 / ε4	3	2 %	6	4 %
ε3 / ε3	83	56 %	70	51 %
ε 3 / ε4	40	27 %	42	31 %
ε4 / ε4	4	3 %	4	3 %
Σ	147		137	
	χ 2=1,41 3 df p=0,70 (rare events (2/2, 2/4, 4/4) were here pooled for the χ2 test)			
-219 (Th1)				
	PD patients		Controls	
	n	f	n	f
T	116	39 %	114	42 %
G	178	61 %	160	58 %
Σ	294		274	
	χ 2=0,27 1 df p= 0,60			
TT	21	14 %	21	15 %
TG	74	50 %	72	53 %
GG	52	35 %	44	32 %
Σ	147		137	
	χ 2=0,34 2 df p=0,84			
+113 (IE1)				
	PD patients		Controls	
	n	f	n	f
C	70	24 %	68	25 %
G	224	76 %	206	75 %
Σ	294		274	
	χ 2=0,08 1 df p=0,78			
CC	10	7 %	6	4 %
CG	50	34 %	56	41 %
GG	87	59 %	75	55 %
Σ	147		137	
	χ 2=1,88 2 df p=0,39			

Table 7. APOE haplotypes in ε3 homozygous PD patients and controls (unpublished data)

	Patients		Controls	
-219G/T- ε3haplotypes	n	*f*	N	*f* %
T- ε3	55	33 %	46	33 %
G- ε3	111	67 %	94	67 %
Σ	166		140	
	χ^2=0,00 1 df p=0,96			
-219- ε3genotypes	n	*f*	n	*f*
T- ε3 / T- ε3	10	12 %	6	9 %
T- ε3 / G- ε3	35	42 %	34	49 %
G- ε3 / G- ε3	38	46 %	30	43 %
Σ	83		70	
	χ^2=0,86, 2 df, p=0,65			
+113G/C- ε3haplotypes	n	*f*	n	*f*
C- ε3	55	33 %	45	32 %
G- ε3	111	67 %	95	68 %
Σ	166		140	
	χ^2=0,03, 1 df, p=0,85			
+113- ε3 genotypes	n	*f*	n	*F*
C- ε3 / C- ε3	10	12 %	5	7 %
C- ε3 / G- ε3	35	42 %	35	50 %
G- ε3 / G- ε3	38	46%	30	43 %
Σ	83		70	
	χ^2=1,51, 2 df, p=0,47			

2.2 *COMT* polymorphisms in PD

The roles of COMT enzyme in catechol (including dopamine) metabolism and detoxication prompted us to study the possible association of *COMT* gene with PD. Val158Met polymorphism encoded by a G to A polymorphism in the corresponding codon in exon 4 of *COMT* gene determines the high and low activity forms of COMT enzyme [280].

No association of this Val158Met and PD was found: alleles and genotypes were equally distributed in PD and control groups (Table 8). We also studied another coding polymorphism rs6267 (Ala72Ser). An association was not found with this polymorphism (Table 8). Rs6270 was not polymorphic in our patients or controls.

COMT gene has been studied as an interesting candidate gene in PD because of its biological function. The results of its association with PD are conflicting [227, 229, 231, 282]. The fact that some studies have found Met allele and others Val to associate with PD, while many like us have found no association, is consistent with the conclusion that these COMT variations likely have no role in PD susceptibility. COMT Val158Met polymorphism's impact on treatment response to antiparkinsonian medication has also been studied, but no effect has been detected [377-380].

Table 8. COMT Val158Met and rs6267 polymorphisms in PD and controls

	PD patients	Controls		PD patients	Controls
COMT alleles	N (%)	N (%)	COMT genotypes	N (%)	N (%)
Val / Met(G / A)					
Val	131 (45 %)	133 (49 %)	Val / Val	30 (20 %)	31 (23 %)
Met	163 (55 %)	141 (51 %)	Val / Met	71 (48 %)	71 (52 %)
Σ	294	274	Met / Met	46 (31 %)	35 (26 %)
χ2=0,9 1 df, p=0,34			Σ	147	137
			χ 2=1,16, 2 df, p=0,56		
rs6267			rs6267		
G	284 (97 %)	263 (96 %)	GG	137 (93 %)	126 (92 %)
T	10 (3 %)	11 (4 %)	GT	10 (7 %)	11 (8 %)
Σ	294	274	TT	0	0
			Σ	147	137
χ 2=0,15 1 df, p=0,70			*χ 2=0,16, 1 df, p=0,69*		

2.3 *Parkin* coding polymorphisms in PD

Parkin mutations are the most common cause for young-onset recessive PD. More than twenty different mutations have been found in this large gene with 12 exons spanning about 1.3 Mb of genomic DNA. Associations of coding polymorphisms of parkin to PD susceptibility have been suggested in Asian and European populations [203, 207]. In Finland, 22 patients with familial PD have been screened for eight parkin mutations but no mutations have been found [202]. Coding polymorphisms of parkin have not been previously investigated in Finnish patients.

Wang et al [203] reported first in 1999 parkin polymorphisms in Japanese sporadic PD. In their study the frequency of Arg366Trp Trp-allele was significantly (1.2 vs 4.4 %) lower in patients than in controls. This polymorphism was expected to alter the conformation of the parkin protein from α-helix to β-sheet, and thus possibly affect the protein function. Ser167Asn and Val380Leu were not associated with PD in that study.

We studied the same coding polymorphisms in our Finnish material, but no associations with PD were found. Arg366Trp (nt1197C→T in exon 10) was not at all polymorphic in our cases and controls. The allele and genotype frequencies of Ser167Asn (nt601G→A in exon4) and Val380Leu (nt1239G→C in exon 10) were similar in cases and controls (all p-values >0,50, Table 9). In our 11 EOP patients with AAO ≤ 45 y the frequency of C-allele (Leu380) was higher than in the rest of our patients and controls (41% vs. 15 and 17.5%). However, this is probably a chance finding due to a small N of our EOP group. A European study following ours has found association of Val380 and sporadic PD [207], and Leu380 has been discussed as possibly protective. Results in a later European study have supported the possible protective effect of Leu380 [201]. Val380Leu has been associated with sporadic PD in a Central European study, but only in early-onset cases, and the associated allele has not been clearly specified [381]. Even though in Asian populations Asn167 has shown association

to PD [382, 383], no European study has found association between Ser167Asn and PD [201, 204, 207, 261, 381]. Thus, these polymorphisms do not seem to have any major role role in the susceptibility to sporadic PD in Finland. However, the possible role of Val380Leu in predisposition to PD needs further study, especially in EOP patients.

It is of note that parkin allele frequencies differ remarkably between Asians and Caucasians, also demonstrated by a recent study that included a meta-analysis of the frequencies of these polymorphisms [384]. For example, Asn167 is present in about 40% of Asians [203, 385], but substantially more rare in our Finnish subjects (0.5-1%) and in other Europeans (~2%) [207, 261], highlighting the importance of cases and controls being ethnically matched.

Table 9. Parkin coding polymorphisms in PD patients and controls

	PD patients	Controls		PD patients	Controls
Parkin alleles	N (%)	N (%)	*Parkin* genotypes	N (%)	N (%)
Ser167Asn(G/A)			Ser167Asn(G/A)		
G	291 (99 %)	272 (99 %)	GG	144 (98 %) *	135(98,5%)
A	3 (1 %)	2 (1 %)	GA	3 (2 %)	2 (1,5 %)
Σ	294	274	AA	0	0
*P=0,53 (*Fischer's exact test*)*			Σ	147	137
			*p=0,53 (*Fischer's exact test*)*		
Val380Leu(G/C)			Val380Leu(G/C)		
G	250 (85 %)	226(82,5%)	GG	106 (72 %)	94 (69 %)
C	44 (15 %)	48 (17,5 %)	GC	38 (26 %)	38 (28 %)
Σ	294	274	CC	3 (2 %)	5 (4 %)
			Σ	147	137
χ^2=0,68, 1df, p=0,41			*χ^2=0,87, 2df, p=0,65*		

* Note that allele frequencies of Ser167Asn are correct here but opposite (incorrect) in II

3. *DJ-1* IN SPORADIC PD (III)

In 2003, Bonifati et al reported two loss-of-function mutations in DJ-1 to be the cause for PARK7, a rare form of autosomal recessive early-onset parkinsonism [155]. A large deletion segregated with PD in a Dutch family, and a homozygous missense mutation in exon 7 (L166P) in an Italian family.
As variation in the genes of familial PD may also affect susceptibility to the more common sporadic PD, we chose to test association of DJ-1 and PD in our case-control material. Because one line of hypotheses on the pathogenesis of PD involves oxidative stress, the putative role of DJ-1 in oxidative stress protection also makes it an interesting candidate gene. Common variation within the promoter regions of *parkin* and α-*synuclein* are suspected to increase the risk for PD [348, 386]. Therefore, of particular interest was an 18 bp insertion/deletion variant in the promoter region of *DJ-1* (g.168_185del). We also tested polymorphisms in the promoter and intron 5 of *DJ-1* in 95 North American samples to study LD across the DJ-1 gene.

No significant differences were found in the allele and genotype frequencies between the patients and the controls (Table 10). The distribution of genotypes was at Hardy-Weinberg equilibrium. Linkage disequilibrium (LD) was observed between the alleles of the g.168_185del and g.213G>T promoter polymorphisms (D' = 0.98 χ^2=201.4 1 df p< 0.0001) and between the promoter (g.168_185del) and IVS5+30T>G in intron 5 (D'=0.76 χ^2=39.5 1 df p< 0.0001).

Table 10. DJ-1 Exon 1 Ins/Del 168_185 polymorphism in PD and controls

	PD PATIENTS n* (%)	CONTROLS n* (%)
ALLELES		
Ins	187 (69 %)	182 (71 %)
Del	85 (31 %)	76 (29 %)
	$\chi^2 = 0.20$, 1 df, $p=0.65$	
GENOTYPES		
Ins / Ins	64 (47 %)	63 (49 %)
Ins / Del	59 (43 %)	56 (43 %)
Del / Del	13 (10 %)	10 (8 %)
	$\chi^2 = 0.29$, 2 df, $p=0.86$	

*Genotyping was not successful in 11 patients and 8 controls.

g.168_185del, a deletion variant within the promoter region of *DJ-1* could be potentially functional due to its localization 168 bp after the transcriptional activation site [387]. However, we did not find any association between this polymorphism and PD. Since strong LD was observed across the gene, it seemed unlikely that analysis of further polymorphisms would produce a positive association with a common predisposing allele in our sample from the homogenous Finnish population.

Our DJ-1 analysis was performed very soon after the report by Bonifati et al [155]. We provided preliminary evidence that common variation within *DJ-1* does not act as a risk factor for PD, but recommended that comprehensive analyses of variation within *DJ-1* in several populations would be required in order to fully assess the role of this gene in the predisposition to sporadic PD. Since then, other studies with other populations have not found an association between *DJ-1* and sporadic PD [102, 199, 200, 388]. Since *DJ-1* mutations among familial and early-onset PD are very rare [102, 199], the overall contribution of *DJ-1* in PD susceptibility seems to be small.

4. *POLG1* VARIANTS IN PD (IV-V)

A considerable amount of evidence has proposed that mitochondria play a role in the pathogenesis of PD [298]. Mutations in the gene *POLG1* encoding mitochondrial DNA polymerase gamma (POLG) lead to secondary defects in mtDNA and subsequent defects and deficiency of the respiratory chain. Parkinsonism co-segregated with *POLG1* mutations in families with progressive external ophthalmoplegia (PEO) [316]. Early-onset parkinsonism in two sisters, who had a history of migraine and anxiety, was also found to result from compound heterozygous POLG mutations. In addition to parkinsonism, these patients had axonal, predominantly sensory neuropathy, but not PEO [320]. These findings encouraged us to study the possible role of *POLG* gene in sporadic idiopathic PD.

POLG1 polyglutamine tract variants in Finnish PD patients

In POLG polyglutamine (polyQ) tract, encoded by a CAG repeat in exon 2, the most common allele lengths are 10CAG (denoted here 10Q, 86-90%) and 11Q (6-9%) [389-391]. These two most common repeat lengths were defined as the "normal" reference alleles and their frequency was compared to the frequency of all other alleles (denoted as non-10/11Q alleles).

The polyQ alleles and their frequencies are presented in table 11. We found significantly increased frequency of exon 2 polyQ tract non-10/11Q length variants (6Q-9Q, 12Q-14Q, 4R+9Q) in our PD patients (10%) compared with our spouse controls (3.5%; p=0.003), and population controls (4.3 %; p=0.001). Thus, the non-10/11Q alleles were clearly overrepresented in the PD patient group. Our data indicates that POLG polyQ variation may influence predisposition to PD.

Previously a small study with 22 PD patients failed to find association between POLG polyQ repeat and PD [226]. The shortcomings of their study were a material too small to exclude association of a gene and a multifactorial disease, and polyacrylamide analysis of the repeat length, which fails to detect polyQ alleles with amino acid substitutions, such as 4R+9Q (=Q43R). In another study, analyzing polyQ and selectively exons 7,8,16,18, and 21 in 140 PD patients, no PD-specific changes were found [225]. However, they also used electrophoresis for polyQ analysis and dHPLC for part of sequence analysis, both of which may fail to detect some sequence changes.

Table 11. *POLG1* exon 2 polyQ alleles in Finnish PD patients and controls

PolyQ allele		PD PATIENTS n=140		SPOUSE CONTROLS n= 128		POPULATION CONTROLS n=516	
		n	f (%)	n	f (%)	n	f (%)
RRRQQQQQQQQQQQQQQQQQPQQPQ	14Q					2	0.2
RRRQQQQQQQQQQQQ-QQQPQQPQ	13Q	2	0.7				
RRRQQQQQQQQQQQ--QQQPQQPQ	12Q	7	2.5	3	1.2	16	1.5
RRRQQQQQQQQQQ---QQQPQQPQ	11Q	15	5.4	15	5.9	88	8.5
RRRQQQQQQQQQ----QQQPQQPQ	10Q	237	84.6	232	90.6	900	87.2
RRRQQQQQQQQ-----QQQPQQPQ	9Q	13	4.6	5	1.9	17	1.6
RRRQQQQQQQ------QQQPQQPQ	8Q	3	1.1			4	0.4
RRRQQQQQQQ-------QQQPQQPQ	7Q						
RRRQQQQQQ--------QQQPQQPQ	6Q	1	0.4	1	0.4	3	0.3
RRRRQQQQQQQQ----QQQPQQPQ	4R+9Q	2	0.7			2	0.2
	Σ	280		256		1032	

POLG1 haplotype analysis

Four-locus analysis with markers rs2238297 C/T, L752L C/T, rs2302084 A/G and rs3087374 G/T (Q1236H) found six *POLG* haplotypes with a frequency > 1%. These haplotypes were not significantly associated with PD. Their frequencies in the PD/control groups were 0.50/0.47, 0.31/0.31, 0.10/0.08, 0.04/0.04, 0.03/0.04,and 0.04/0.02 (global $p > 0.1$; individual haplotype p-values > 0.08). We analyzed the four above-mentioned SNPs together with the CAG-repeat alleles to test whether the association of the CAG repeat (polyQ) alleles with PD is influenced by their haplotype background.

One haplotype (9Q-CCAG) had an increased frequency among PD patients compared to spouse controls (4.9% vs. 1.3%). In SNPHAP analysis this particular 9Q-haplotype was found in all 13 patients who carried 9Q and in 3 spouse controls out of 5 carrying 9Q ($\chi 2 = 4.93$, 1 *df*, nominal $p = 0.026$), while 9Q-allele alone, ignoring haplotype background, did not show a difference ($\chi 2 = 2.49$, 1 *df*, $p = 0.11$). The significance of this haplotype finding remains uncertain due to the small number of these 9Q haplotypes; it may be a chance finding and needs to be confirmed in other studies.

POLG1 missense variants

A novel missense mutation S1230F was found in one PD case, but in none of the spouse controls. However, the patient's 95-year-old sister who does not have PD had the same mutation, suggesting that it is a neutral variant. Three previously reported common coding-region polymorphisms (Q1236H, E1143G, K317K) were found in similar frequencies in PD patients and spouse controls (all p-values >0.2).

Y831C mutation has previously been reported as the possible culprit in an Italian family with parkinsonism, hypogonadism, and neuropathy [317]. Y831C was present in one of our PD patients but also in 5 controls. The controls carrying this variant were born in years between 1924 and 1946 and already have advanced age but have not had neurological symptoms requiring contact to neurological health services, except one woman who was diagnosed with tension type headache. Therefore, our results argue against Y831C's pathogenic role [317]. It is likely that Y831C is a neutral polymorphism.

Clinical features of the patients with *POLG1* variants

Our patients were collected to represent idiopathic sporadic PD, excluding atypical and familial cases. None of our 14 patients carrying rare non-10/11Q alleles, missense variants, or both had eye movement abnormalities or ptosis, which are typical features of *POLG1*-associated PEO-disease. One patient carrying 4R+9Q (Q43R), whose initially typical parkinsonism presented at the

age of 54, later developed an atypical syndrome including severe laryngospasms, urinary incontinence, cervical dystonia and brisk reflexes. He died at the age of 62 years. Other patients have not shown atypical features in 7-15 years follow-up from the onset of PD symptoms.

Even though several studies have now reported parkinsonism as a part of the disease spectrum in *POLG*-associated disease with PEO-type features, ataxia, or neuropathy [316, 320-322], association of *POLG* variation to idiopathic PD has not been previously found. The present data support a role for POLG polyQ variants in idiopathic sporadic PD in Finland. To assess the global role of POLG in PD, studies in other populations are needed.

POLG polyQ variation in North American PD patients and controls (V)

Our intriguing results showing increased amount of rare non-10/11Q POLG polyQ length variants in Finnish PD patients compared to controls prompted us to carry out a replication study on the polyQ variation in a larger sample of North American PD patients (n=652) and controls (n=292). PolyQ allele and genotype frequencies were similar in early (≤45 years) and late-onset patients (AAO > 45 years). Neither were polyQ alleles or genotypes in patients with a positive family history for PD different from those patients without (Data not shown). The whole patient material was therefore used for comparisons with the controls. The *POLG1* polyQ genotypes are presented in table 12. Allele frequencies of the US PD patients and US controls, and for comparison, literature controls are presented in table 13.

Table 12. *POLG1* exon 2 polyQ genotypes

	US PD PATIENTS N=652		US CONTROLS N=292	
PolyQ GENOTYPE	n	f (%)	n	f (%)
6Q/11Q	1	0,2	0	0
7Q/10Q	7	1,1	0	0
7Q/11Q	1	0,2	1	0,3
7Q/12Q	1	0,2	0	0
7Q/14Q	1	0,2	0	0
8Q/10Q	5	0,8	1	0,3
8Q/11Q	0	0	1	0,3
9Q/10Q	11	1,7	7	2,4
9Q/12Q	1	0,2	0	0
10Q/10Q	443	67,9	225	77,1
10Q/11Q	133	20,4	44	15,1
10Q/12Q	31	4,8	10	3,4
11Q/11Q	6	0,9	1	0,3
11Q/12Q	4	0,6	2	0,7
12Q/12Q	2	0,3	0	0
4R+9Q/10Q	4	0,6	0	0
4R+9Q/11Q	1	0,2	0	0

Based on previous literature on allele frequencies and our previous study on Finnish patients, lengths 10Q and 11Q were considered common, neutral alleles, and other alleles as rare variants (non-10/11Q), and the frequencies of the 10/11Q and non-10/11Q alleles were compared in PD patients and controls. Non-10/11Q alleles (6Q, 7Q, 8Q, 9Q, 12Q, 13Q, 14Q, and 4R+9Q) were more common in PD patients (5.8 %) than in controls (3.8 %), in accordance with our previous study on Finnish patients. However, this difference did not reach statistical significance (two-tailed p=0.07; OR 1.6, 95% CI 0.96-2.53). Thus, our hypothesis based on the previous Finnish results was not confirmed at a statistically significant level. Comparison to a larger set of 1541 controls confirmed the lack of significant association in this setting (table 14).

Table 13. POLG exon 2 polyQ alleles in the present study and in literature controls

	THE PRESENT STUDY		LITERATURE CONTROLS		
	US PD PATIENTS n=652	US CONTROLS n=292	FINNISH CONTROLS* n=644	MIXED CAUCASIAN CONTROLS # n=605	ALL CONTROLS POOLED n=1541
PolyQ allele	n (%)	n (%)	n (%)	n (%)	n (%)
6Q	1 (0,1)	0 (0)	4 (0,3)	0 (0)	4 (0,13)
7Q	10 (0,8)	1 (0,2)	0 (0)	2 (0,17)	3 (0,10)
8Q	5 (0,4)	2 (0,3)	4 (0,3)	5 (0,41)	11 (0,36)
9Q	12 (0,9)	7 (1,2)	22 (1,7)	22 (1,8)	51 (1,7)
10Q	1077(82,6)	512 (87,7)	1132 (87,9)	1045 (86,4)	2689 (87,2)
11Q	152 (11,7)	50 (8,6)	103 (8,0)	99 (8,2)	252 (8,2)
12Q	41 (3,1)	12 (2,1)	19 (1,5)	28 (2,3)	59 (1,9)
13Q	0 (0)	0 (0)	0 (0)	4 (0,33)	4 (0,13)
14Q	1 (0,1)	0 (0)	2 (0,15)	1 (0,08)	3 (0,10)
4R+9Q	5 (0,4)	0 (0)	2 (0,15)	4 (0,33)	6 (0,19)
Σ	*1304*	*584*	*1288*	*1210*	*3082*

* pooled spouse and population controls from IV

\# from the doctoral thesis of Rovio A 2006 http://acta.uta.fi/english/teos.phtml?10863

Previous literature on the phenotypic effects of *POLG1* polyQ has mostly defined only the 10Q as the normal reference allele [390, 392]. Therefore, we also analysed whether the frequency of the non-10Q alleles (including also 11Q) in the PD patients differs from the controls. The 10Q/10Q genotype was significantly less common in PD patients than in controls (67.9% vs. 77.1%), and accordingly, other genotypes more common in the patient group (32.1% vs. 22.1%, χ^2=8.09, 1 df, p=0.004). The frequency of the non-10Q alleles was increased in the PD patients compared to the controls (17.4% in the US PD group vs. 12.3% in US controls; χ^2=7,81, p=0.005; OR 1.50, 95% CI 1.13-1.99). The association with the non-10Q alleles and PD was also significant in the more powerful analyses when compared to the literature controls (table 14.)

Table 14. Association of *POLG1* polyQ alleles with PD

	US PD n=652	US CONTROLS n=292	FINNISH CONTROLS# n=644	MIXED CAUCASIAN CONTROLS* n=605	ALL CONTROLS POOLED n=1541
Alleles	n (%)	n (%)	n (%)	n (%)	n (%)
10Q+11Q	1229 (94.2)	562 (96.2)	1235 (95.9)	1144 (94.5)	2941 (95.4)
Non-10/11Q	75 (5.8)	22 (3.8)	53 (4.1)	66 (5.5)	141 (4.6)
Σ	1304	584	1288	120	3082
		χ^2=3.26, p=0.07	χ^2=3.70, p=0.06	χ^2=0.10, p=0.75	χ^2=2.71, p=0.10
Alleles					
10Q	1077 (82.6)	512 (87.7)	1132 (87.9)	1045 (86.4%)	2689 (87.2)
Non-10Q	227 (17.4)	72 (12.3)	156 (12.1)	165 (13.6)	393 (12.8)
Σ	1304	584	1288	1210	3082
		χ^2=7.81, *p=0.005*	χ^2=14.43, *p=0.0001*	χ^2=6.78, *p=0.009*	χ^2=16.37, *p=0.00005*
Genotypes	n (%)	n (%)			
10Q/10Q	443 (67.9)	225 (77.1)			
Other genotype	209 (32.1)	67 (22.9)			
Σ	652	292			
		χ^2=8.09,*p=0.004*			

"Normal" allele defined either as 10Q + 11Q or 10Q only; variant alleles non-10Q include 6Q-9Q, 11Q-14Q and 4R+9Q; variant alleles non-10/11Q include 6Q-9Q, 12Q-14Q and 4R+9Q.
χ^2 and p are from comparisons between the US PD group and the control groups
* from the doctoral thesis of Anja Rovio http://acta.uta.fi/english/teos.phtml?10863 , p.60
# pooled spouse and population controls from IV

Typically in polyQ–associated diseases the repeat expansion is pathogenic. However, the CAG repeat length variation in the androgen receptor gene is an example demonstrating that even short and long alleles within the normal range may be associated with different disease phenotypes [393]. The functional effects of the length variation in *POLG1* polyQ repeat are not known, but the polyQ of *POLG1* is relatively stable, as over 80% of people carry the most common length 10Q [390, 391]. Also in primates the repeat length is highly conserved, although its exact length is species-specific, proposing that the length of the tract may be important for POLG function [390].

Our results indicate that non-10Q variant length alleles of the *POLG1* polyQ tract may increase predisposition to PD, and should motivate more research in this field. Sequencing-based analysis of *POLG1* polyQ repeat in genetic association

studies in other populations and materials large enough, as well as studies on the functional consequences of the POLG polyQ variants are required.

CONCLUDING REMARKS AND FUTURE PROSPECTS

In this thesis, the usefulness of ^{123}I-β-CIT SPECT in the routine diagnosis of PD was first evaluated. Genetic risk factors for sporadic PD were studied in a set of PD patients and their spouses as controls. ^{123}I- β -CIT SPECT was used as a part of patient selection to the genetic study.

Allelic association was not found between sporadic PD and *parkin* (PARK2) or *DJ-1* (PARK7), mutations in which cause autosomal recessive parkinsonism.
The functional Val158Met polymorphism or another coding polymorphism of *COMT* were not associated with the risk of PD. This adds to the conflicting, mainly negative literature reports.
We are the first to report APOE promoter and enhancer region polymorphisms –219G/T and +113G/C and *APOE* ε3 haplotypes in PD. No association with PD was found. The *APOE* ε2/3/4 polymorphism was not associated with PD susceptibility either, even though it modifies risk for Alzheimer's disease and prognosis of for example brain trauma.

An association was found between PD and *POLG1*. First, in our Finnish sample we found increased amount of rare non-10/11Q length variants of *POLG1* exon2 CAG repeat (polyQ) in PD patients compared to controls (10% vs. 3.5%, p=0.003). The two most common lengths 10Q and 11Q were initially considered as the normal reference lengths and alleles 6Q-9Q, 12-14Q and 4R+9Q as rare length variants, since the summarized allele frequency of 10Q (86-90%) and 11Q is in different populations around 95 %. Our positive result in Finnish patients motivated us to perform a larger replication study on *POLG1* polyQ length variation in US PD patients and controls. This study with a higher statistical power study led to sharpening the definition of the "normal" allele. Also other studies on other phenotypical phenomena and *POLG1* mainly considered 10Q as the only "normal" allele. The replication study did not confirm the pathogenic role of the non-10/11Q alleles, but showed that non-10Q alleles (including 11Q) were significantly more common in patients than in controls (p=0.005). Comparison to other control groups also showed significant association with PD (p=0.00005).

Our results strongly suggest that alleles other than 10Q may increase susceptibility to PD. Mutations in POLG may lead to secondary defects in mtDNA, and thus mitochondrial dysfunction, suspected in the pathogenesis of PD. Functional effects of the polyQ length variation are not known. Large replication studies are warranted, in addition to studies on the functional consequences of the *POLG1* polyQ variants.

Earlier studies had reported ^{123}I-β-CIT SPECT in selected sets of patients with a specific movement disorder compared to controls. We studied how useful ^{123}I-β-CIT SPECT is in the differential diagnosis of PD in a consecutive real-life material including various movement disorder patients. Our results show that ^{123}I-β-CIT SPECT may aid in the diagnosis of PD, differentiate between PD and DIP, ET, psychogenic parkinsonism, and those cases of VP who do not have a striatal vascular lesion. P+ and DLB patients had reduced uptake similar to that of PD patients, and thus ^{123}I-β-CIT SPECT cannot help in differentiating these conditions from PD. However, in the differential diagnosis between DLB and Alzheimer's disease ^{123}I-β-CIT SPECT may be helpful. ^{123}I-β-CIT SPECT correlated with symptoms rather than age and since it was able to detect presynaptic nigrostriatal defect already in asymptomatic phase (bilateral decrease in uptake in patients with unilateral PD), ^{123}I-β-CIT SPECT may be used to screen presymptomatic PD among at-risk individuals.

Since already 60 % of the nigrostriatal neurons are lost at the time of PD diagnosis, an important challenge is to shift PD diagnosis to an earlier phase of the disease, especially in the future, when hopefully neuroprotective therapies will be available. ^{123}I-β-CIT SPECT may be one way to detect PD in presymptomatic (or at least premotor) stage in at-risk individuals. Also screening those subjects with possible premotor features of PD, such as hyposmia or REM sleep behavioural disorder, might be beneficial. Clinical studies are needed to recognize more symptoms or signs that may precede PD.

So far no biomarkers of PD are available. A future means to detect early PD might be measuring gene expression signals in the blood. Recently, a study on

early-stage PD patients found significantly reduced or increased expression in 22 genes in a whole-genome expression chip analysis. Remarkable part of these genes were involved in the processes relevant in PD, e.g protein folding, ubiquitin-proteasome system and mitochondrial function [394].

During the years that this thesis was carried out, research and knowledge on the genetics of PD expanded. Genetic studies have revealed new metabolic routes in the pathogenesis of PD, for example altered function or amount of SNCA, disturbances in protein degradation, especially ubiquitination (UCHL1, parkin), and impaired mitochondrial function (PINK1, parkin, HTRA2/Omi, DJ1, and our results on *POLG1*). It is likely that PD results from many different genetic susceptibility variants with small individual effect interacting with environmental factors. Hope of finding a single common pathway of pathogenesis for neurodegeneration in PD may be illusory. PD may not be just one disease: instead, there may be different neurometabolic subgroups finally leading to similar clinical and neuropathological outcome. Recognizing these subgroups would be important in developing preventive or neuroprotective therapy. Hopefully, in the future, tailor-made neuroprotective therapy could be offered for each patient according to the patient's PD subtype.

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APPENDIX

Candidate genes studied in sporadic PD (random order)

1. LRRK2
2. NAT2
3. MTHFR
4. PINK1
5. SCA2
6. MTH1
7. SNCA
8. IL10
9. POLG=POLG1
10. SNCA multipl
11. CYP2D6
12. MAPT =tau
13. ATP13A2
14. GBA
15. UCHL1
16. MTIF1
17. GSTP1
18. GSTM1
19. APOE
20. BDNF
21. LRRK1
22. CARD15/NOD2
23. DJ1
24. NR4A2=Nurr1
25. GRK5
26. POMC
27. SIAH1
28. parkin
29. GCH1
30. TH
31. Sema5A
32. SLC18A2=VMAT2
33. SPR=Sepiapterin
34. CD14
35. FGF20
36. α1antichymotrypsin=SERPINA3
37. TATA box binding protein
38. Synaptotagmin XI
39. PON1
40. FMR1=FRAXA
41. MAO-A
42. MAO-B
43. NEUROFILAMENT M
44. MCP1 =CCL2
45. CCR-2
46. SCA17
47. SYNPHILIN-1=SNCAIP
48. DAT=DAT1=SLC6A3
49. IRP2=IREB2
50. COMT
51. NEUROFILAMENT L =NEFL
52. NFKB1
53. A2Macroglobulin=A2M
54. ACE
55. hSKCa3
56. TNF-a
57. DRD5
58. IL6
59. TNFR1
60. DCP1
61. BCHE
62. IL-1B
63. IL-1A
64. IL-1R
65. CYP1A1
66. persyn=SNCG=synuclein γ
67. MnSOD=SOD2
68. mt tRNA a4346G
69. DRD2
70. ND2
71. Cu/ZnSOD
72. CAT=catalase
73. DRD3
74. DRD4
75. CYP2E1
76. HTRA2/OMI
77. iNOS =NOS2A
78. PRNP
79. TYROSINASE=TYR
80. ADH1C
81. GSTO1
82. GSTZ1
83. GSTO2
84. DRD1
85. PANK2
86. PASD1
87. ND5
88. ND1
89. ND3
90. ND4
91. SCA8
92. HLA
93. SCA3
94. IFNg
95. EPHX1
96. EPHX2
97. NQO1
98. NQO2
99. 5HTT=SCL6A4
100. ND6
101. CCK
102. ACO2
103. GLO-1
104. PARP1
105. MTR
106. TGFB2
107. PTGS2
108. GCT25D1

109. NDUFS2
110. NGFB
111. NEGR1
112. ELAVL4
113. CDCP2
114. HTR6
115. PRDM2
116. CYP1B1
117. HNMT
118. ABP1
119. IL1RN
120. PITX3
121. LMX1B
122. OTX2
123. EN1
124. GALNT3
125. NDUFS1
126. USP24
127. USP40
128. RANTES =CCL5
129. CCR5
130. TF =transferrin
131. TFR1
132. HFE
133. FRATAXIN
134. LACTOFERRIN
135. GAP43
136. GSK3B
137. PRKCZ
138. EDEM1
139. VIPR1
140. SIAH2
141. CP =ceruloplasmin precursor
142. WFS1
143. CCKAR
144. PHOX2B
145. ASCL1
146. IL8
147. IL2
148. ADH1B
149. ADH4
150. CASP6
151. ANK2
152. FGF2
153. MGST2
154. CASP3
155. DAP
156. NDUFS4
157. ACSL6
158. HSPA6
159. CSNK1A1
160. HRH2
161. SNCB
162. GRK6
163. CANX
164. CSNK2B
165. HSPA1L
166. HSPA1A
167. HSPA1B
168. VEGFA
169. PLA2G7
170. GSTA4
171. CNR1
172. FABP7
173. ESR1
174. SLC22A1
175. SLC22A2
176. SLC22A3
177. OGDH
178. DDC
179. ABCB1=MDR1
180. PON2
181. DLD
182. CAV1
183. GPR37=PAELR
184. PTN
185. NOS3
186. eNOS
187. nNOS = NOS1
188. EN2
189. SHH
190. NAT1
191. CTSB
192. NDUFB9
193. DNAJA1
194. VCP
195. UBQLN1
196. CTSL
197. NDUFA8
198. TOR1A
199. DBH
200. TFAM
201. CYP2C9
202. CYP2C19
203. SCD
204. NDUFB8
205. GBF1
206. CTSD
207. IGF2
208. INS
209. CCKBR
210. CALCA
211. CNTF
212. FADS1
213. FADS3
214. FTH1
215. CTSF
216. ABRBK1=GRK2
217. NDUFS8
218. PHOX2A
219. RAB6A
220. MMP1
221. MMP3
222. NCAM1
223. HSPA8
224. GRIN2B=NMDAR2B
225. LRP1=A2MR
226. APAF1

227. MTIF3
228. HTR2A
229. ESR2=ESRB
230. MTHFD1
231. CTSH
232. CYP1A2
233. CSNK2A2
234. HP (haptoglobin)
235. FA2H
236. STX8
237. ACACA=ACC1
238. SUMO2
239. P4HB
240. ADCYAP1
241. CDH2
242. BCL2
243. NDUFS7
244. NDUFA7
245. ICAM1
246. CALR
247. NDUFB7
248. CPAMD8=VIP
249. FTL
250. PRKCG=SCA14
251. CSNK2A1
252. CHRNA4
253. PSMA7
254. SS18L1
255. IFNGR2
256. ADORA2A
257. KCJN6
258. GSTT1
259. ADRBK2
260. YWHAH=14-3-3 eta
261. HMOX1
262. SEPT3
263. NDUFA6
264. NDUFA1
265. FRAXE=FMR2